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Histological Studies on the Mode of Penetration of Boll-Rotting Organismsinto Developing Cotton Bolls.

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HISTOLOGICAL STUDIES ON THE MODE OF PENETRATION OF BOLL-ROTTING ORGANISMS INTO DEVELOPING COTTON BOLLS

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ABSTRACT

A study of cotton boll tissues involved in dehiscence was made to determine their role in initiation of boll rot. Gossypium hirsutum L. varieties 'Stoneville 7A' and 'DPL 16' were used. Transverse sections were made of external sutural regions of field-grown bolls 5-50 days of age from anthesis. Anatomical features of external sutural areas were described, and dehiscence was followed through progressive boll ages. Initial sutural splitting occurred between the 10th-15th day near the sutural center and proceeded centripetally and centrifugally to completion. This indicated that sutural dehiscence began before external boll maturity was complete. On approximately the 45th day, boll exocarps were completely separated, exposing the locules.

Paradermal and transverse sections of pericarps of 5-55 day old, apparently healthy field-grown bolls were made to investigate entry of boll-rotting organisms. These sections revealed fungal spores and/or mycelia randomly covering bolls after the 5th day. As boll age increased to the 20th-25th day, marked changes in stomatal openings were noted. Guard cells of 20-25 day bolls apparently became non-functional or senescent, stomata remained open, and were penetrated by hyphae of various fungal species.

Fungi colonized the outer pericarp tissues and entered the sutural areas following progressive dehiscence. There was a correlation between the time of stomatal penetration and initial boll dehiscence. Sutural colonization by rot organisms was followed by fiber deterioration in the locules.

Two separate lots, of ten 5-50 day old bolls each, were randomly selected from both Morrow and St. Joseph plots. Peduncles were cut away and bolls surface sterilized in one group, and floral accessories were removed from both lots. Boll rot developed by the 5th day in the nonsterilized group, while little rot was noted before the 20th day of boll age in the sterilized bolls. Callus tissue formed on the cut surface of many of the 5-20 day bolls, indicating a practically microorganism-free interior until the 20th-25th day of boll development.

Field-grown bracts on squares and bolls through dehiscence were selected, individually surface sterilized and aseptically sectioned into apical, middle and basal portions. These portions were plated on water agar and incubated. Results showed potential boll-rot inoculum growing from within the bract portions and indicated a continuous association of boll-rot microorganisms with floral as well as boll stages of development.

Visual presence of microorganisms within bracts 5-45 days of age was demonstrated. Paradermal and transverse bract sections revealed hyphae entering stomata at the apex of bracts and colonizing apical, middle and basal regions, progressively, as bract age increased.

A comparative greenhouse study was undertaken in which the mode of entry and colonization of bracts and bolls by Diplodia gossypina Cke. were determined. Diplodia-spore suspensions were applied to greenhouse bolls and bracts of ages 5-60 days. Constant high humidity

was maintained from misters by day and steam vapor at night. Inoculated bracts and bolls were removed after 5 days and paradermal and transverse sections made of bracts and external boll sutures. Abundant spores and mycelia were found on the surfaces of bracts and bolls, and penetration of both via stomata was observed.

Outer cell layers of pericarps were colonized following stomatal entry between 35-40 days of boll age, mycelia entered progressively dehiscing sutures, and fiber rot began before dehiscence was complete.

D. gossypina entered through the stomata near the bract apices and proceeded to the bases with increasing bract age.

Isolations of Diplodia made from inoculated bracts on all stages from squares to boll dehiscence indicated fungal association with bracts through all periods of their development.

INTRODUCTION

The sequence of events involving changes in the morphology of dehiscing cotton boll sutures of Gossypium hirsutum L. has been generally described (4, 8). However, more specific information as to the precise time and initial point at which sutural dehiscence begins was needed because of its apparent relationship to boll rot development.

There are several areas of the cotton boll which have been described as points of ingress by microorganisms (1, 5, 18). The morphology of the boll is such that the carpel walls are involved in all or most of these possible portals of entry. The possible exception to this would be at the union of the carpel tissues with the receptacle of the boll and the vascular tissue within the receptacle disc.

A detailed study of the boll surface and pericarp tissues was required to determine the actual mode of penetration and colonization used by some microorganisms which come into contact with cotton bolls. This might lead to an explanation for the occurrence of much of the microbial growth taken from the boll apices, bases, fiber in the locules, and the placental columns by other workers (1, 5, 18).

The role of the bract in cotton boll rot development has been established by Luke (12) and Luke and Pinckard (13, 14, 15).

Further investigation of the occurrence of various microorganisms on and within bracts and of the developmental stage of the bract at which this association takes place was believed necessary. If the

boll pathogens could be found within the bracts during the early stages of boll development, then this would demonstrate potential boll-rot inoculum to be available whenever the boll becomes susceptible. This susceptibility could result from natural boll maturation and/or through insect or other injury.

The purpose of this research was to determine the specific developmental process of boll maturity in relation to the mode of penetration of boll-rotting microorganisms into bolls and their colonization of the tissues of the boll pericarps. The time in boll development when microbial inoculum, from bracts and other external sources, becomes available for boll rot was reported. The correlation of the time and mode of boll sutural dehiscence with the period of infection of the pericarp by microorganisms was demonstrated for the first time.

LITERATURE REVIEW

Literature Related to Boll Suture Anatomy and Dehiscence

The cotton boll is a capsule with loculicidal dehiscence (splitting along the plane of the dorsal suture of each carpel). The time of dehiscence depends on varietal differences, dates of blooming, soil fertility, and varying climatic conditions--such as soil moisture, relative humidity, and other factors.

Boll maturation, for the purpose of this study, will mean the interval between flower opening and fruit dehiscence. Cotton bolls are considered dehisced when both the internal and external sutures are split and the fiber in the locules is fully exposed.

The most comprehensive and detailed studies on the tissue anatomy of the boll suture, and especially the developmental morphology of those tissues involved in dehiscence, were presented by Baranov and Maltzev (4), De Coene (7), Gubanob (8), and Joshi et al (11). According to these workers, the dorsal suture, when seen in cross-section, is located in the center of the pericarp between two partitional carpel walls. The tissues which comprise the dorsal suture are the epidermal parenchyma at the outer limits of the suture, the thin-walled parenchyma of the center of the suture which are torn apart during dehiscence, and the tannin-filled parenchyma which border the central parenchyma from the epidermal cells to the locule.

Radial sclerenchyma bundles are associated with the dorsal suture from the epidermal layers to the middle of the mesocarp. Large concentrations of sclerenchyma fibers continue from the midpoint of the mesocarp along both sides of the suture to the sclerotized endocarp.

Cotton boll dehiscence has long been observed. The tissues involved in sutural splitting and their change in morphology have been described (4, 7, 8).

Gubanob (8) assumed that the number and arrangement of the sclerenchyma bundles bordering the dorsal suture had something to do with the dehiscence of the boll.

A constant moisture loss occurs from the pericarp tissues through the stomata as the boll matures, resulting in increased drying of internal boll parts.

Evidence was found by other workers (4) that the arrangement of the sclerenchyma bundles and their drying led to progressive contractions of the cells at the pericarp periphery. This resulted in the turning out of the dehiscing carpel walls and exposure of the fiber in the locks.

The thin-walled parenchyma cells which constitute the center of the dorsal suture were not mentioned as being directly involved in boll dehiscence. Apparently they were considered as having a passive role and were split apart from the force exerted by the contracting and twisting sclerenchyma fibers as they gave up moisture. While studying the line drawings and plates of these workers (4, 7, 8, 11) depicting the tissues of the dorsal suture, it was noted that the mature boll sutures pictured exhibited no evidence of initial dehiscence.

Methods of Boll Penetration by Microorganisms

Boll rot has been recognized since the mid 1800's as a major problem in cotton production in the United States. The boll rot

studies from the 1850's to the mid 1960's were directed to the causes of boll rot, descriptions of the microorganisms and the disease, various methods to control boll rot, and reports on yields affected by boll rot. Very little concern was given to the complex relationship of the disease-causing microorganisms to their host.

Intensive work on the modes of infection by boll-rotting microorganisms of cotton has been observed only since 1964.

The studies of Luke and Pinckard (13) and Luke (12), in 1964 and 1965, respectively, were the first organized attempts at determining the relationship of the boll-rotting organisms with the diseased host (bolls). These workers established for the first time that infected bracts were a primary source of boll-rot inoculum and that these bracts predisposed the bolls to infection. It was found that removing the bracts from field bolls caused a marked reduction in boll rot. This work led to the conclusions that the floral bract was a useless appendage and a major contributor to boll decay (14, 15). Following the work of Luke and Pinckard (13, 14), other researchers have also isolated microbial growth from within surface sterilized field bracts (6, 9).

In work with the boll-rotting organism Pellicularia filamentosa (Pat.) Rogers, Pinckard and Luke (16) found that the fungus directly penetrated the bracts near the base of the boll, entered the base of the boll through the carpel walls and into the placental columns, causing boll decay.

Cauquil and Ranney (5), working on pathogenicity studies of 7 secondary rot organisms, selected field bolls aged 5 to 7 weeks.

The bolls were surface sterilized and inoculated, either by contact with the middle of the valve, contact on the boll apex, or by puncture inoculation of the boll, to show the primary modes of entry. They concluded that the most important avenues of infection are through the apices of the valves, the sutures of the valves, and directly through the carpel wall.

A further study by these workers (5) dealing with organisms found within apparently healthy cotton bolls, revealed additional points of entry into the boll by microorganisms. They excised and surface sterilized 100 field bolls 5 to 7 weeks of age. Isolations were made from the boll apices, placentae, peduncles, insect calluses, rotted seed, calyx pits, and nectaries. They found that the primary areas of entry into bolls were the apex of the boll and sutural leaks, nectarial penetration, and pedicellar infection. Although the term "direct penetration" was used in this work (5), a description of the actual process of infection was not included, nor isolations made, from the pericarp tissue.

Fungal invasion of apparently healthy field bolls was reported by Roncadori (17). The study of succession of fungal growth within the receptacle, boll apex, and locule of field bolls revealed that Glomerella gossypii (Southworth) Edg. colonized the apex most frequently, whereas Ascochyta sp., Fusarium sp., and Gliocladium sp., were associated with the receptacle. Alternaria sp. invaded both apex and receptacle. The ages of the field bolls selected were not stated, although fungi were isolated from the locules of more than 30 percent of the intact, non-decayed bolls.

Apparently healthy green bolls 6-7 weeks of age were selected by Bagga and Ranney (2), to determine the boll-rot potential, organisms involved, and actual boll rot present at the Delta Branch of the Mississippi Agricultural Experiment Station in 1967. They surface sterilized 120 excised bolls for each of 7 cotton varieties and incubated the bolls in jars until boll rot appeared. Isolations and identifications were made of the microorganisms found on the boll surface, and the results showed 44% of the apparently healthy bolls were internally infected.

Roncadori (18), working on the association of boll-rot pathogens with developing bolls, stated that among the many microorganisms isolated Alternaria sp. was the most ubiquitous fungus found from the first week through boll development. This was in agreement with other workers (1, 2, 5). The primary sites of boll infection found by Roncadori were the boll apex, peduncle and locks. It was noted that isolations were made from carpel wall tissue only when lesions were present.

Luke (12), and Luke and Pinckard (14, 15) observed that if the bracts of bolls approaching maturity were removed aseptically, the uninjured bolls did not rot. They reported callus tissue formation on the cut surfaces of many bolls after 6 to 7 days of incubation. They concluded that internal, latent, or systemic infection of uninjured field bolls prior to dehiscence is non-existent or rare.

In a study of the microflora of cotton bolls in Louisiana, Guidroz and Pinckard (9) made collections of bolls "three-quarters grown" during the 1967 cotton season. Each sample consisted of

20-25 bolls from various fields throughout Louisiana. Isolations were made from the bracts, nectaries, boll apices, and placental columns and the microorganisms identified. Their results showed that no microorganisms were found in the placental columns of bolls until very near the time of boll splitting. Callus tissue formation was observed on many of the bolls held in sterile incubation chambers. Similar observations were reported by Pinckard and Luke (16), in which they isolated from the placental columns of hundreds of bolls taken from a state survey and failed to find microorganisms within apparently healthy unopened green bolls. In this same study it was found that microscopic sutural splits occurred in field bolls 35-40 days of age. These openings were considered as prime entry sites for surface inhabiting microorganisms.

The findings of these workers (9, 12, 14, 15, 16) are not consistent with those of Bagga and Ranney (1, 2), Cauquil and Ranney (5), and Roncadori (17, 18). Reasons for the inconsistency of their reports in the literature are not presently known.

A review of the literature on boll penetration and the "boll-rot complex" indicates that most workers have overlooked the time of initial boll dehiscence and its relationship to boll infection (1, 2, 3, 5, 9, 12, 13, 14, 16, 17, 18).

Several researchers (1, 2, 3, 5, 17, 18) apparently did not consider microbial penetration and growth within the major portion of the pericarp between the apex and base of the boll of any importance. Detailed microscopic evidence of actual invasion of primary points of boll entry has not been reported in the literature.

In 1968 Delgado (6), working on the pathology of Diplodia gossypina Cke. in relation to boll rot, was the first to demonstrate boll penetration via the stomata by a fungus. Although he reported the occurrence of stomatal penetration near the time of boll dehiscence and did not report the time of initial boll entry as to boll age, this was the first study in which actual visual proof of boll stomatal penetration was presented. He noted that boll stomata probably provided the primary mode of entry for many boll-rotting organisms. Delgado stated that natural openings or cracks produced as the boll dehisced provided entry for surface inhabiting microorganisms into the locules of the boll.

As a result of Delgado's work it was apparent that detailed histological research on the developing cotton boll in relation to infection was needed. Part of the objective of this research was to provide such a study.

MATERIALS AND METHODS

During 1969 field plots of Upland cotton, Gossypium hirsutum L. varieties 'DPL 16' and 'Stoneville 7A', were established at Morrow and St. Joseph, Louisiana, respectively. Future boll populations were selected by tagging 200 open white blossoms at anthesis, and weekly for 9 weeks at each plot site. The white blossom stage was considered to be day 1 of the initiation of boll development. Bracts were tagged at the beginning of very young square formation through anthesis. They were collected for study from the young square stage through the period of boll dehiscence.

Field Investigations

Boll Studies:

The first part of the study was directed toward showing the progress of boll dehiscence. Field-grown bolls 5-45 days of age were selected from the Morrow plot and fixed in formalin-alcohol-acetic acid (FAA) for microtome sectioning of the external sutural area. Transverse sections of 10-15 microns were made by following a standard paraffin embedding and staining technique prescribed by Jensen (10) and Sass (19). A series of photomicrographs were made of the external sutural anatomy, with identification of the tissues.

In the second part of the study, the mode of entry of boll-rotting organisms into bolls was studied using apparently healthy field bolls 5-45 days old. Ten bolls were selected at random for each 5 days of age from the Morrow plot, and paradermal sections were

made of the exterior of these by freezing the entire bolls (dry) for 36 hours at 10° F, then placing them in water at 140° F for 1-2 hours. The cuticle, epidermis and first layer of subepidermal cells were peeled from the carpel wall, placed on a microscope slide, cuticle side up, stained with 1% safranin, and covered with a cover slip.

Transverse sections of the entire pericarp were also made from apparently healthy Morrow bolls of 5-45 days of age, using the previously mentioned techniques for microtome sectioning. Ten bolls were selected at random for each 5-day age increment. A series of photomicrographs were made of the sections. Prior to sectioning, isolations of microorganisms were made from the internal tissues of the individual pericarps and later compared with any microorganisms found in the stained sections. The isolations were plated on sterile 2% water agar and incubated at room temperature for identification of any microorganisms found.

Ten apparently healthy bolls, differing in age by increments of 5-day intervals from 5-50 days, were also selected at random from both Morrow and St. Joseph plots for additional study. The bracts, sepals, and peduncles were removed, and the bolls were surface sterilized in a 95% ethyl alcohol dip followed by soaking in a 10% Clorox (5.25% sodium hypochlorite) solution for 15 min. The bolls were then rinsed in sterile water and placed, one boll per bottle, in sterile, 80 ml, capped and labeled specimen bottles containing moist filter paper. The bracts and sepals were removed from a similarly selected lot of bolls; however, the peduncles were not removed to avoid introducing surface microorganisms mechanically into the interior of

the boll. Both the sterile and non-sterile groups were incubated at room temperature for 75 days, and observations of microbial growth were recorded at 5-day intervals. After final observations, isolations and identification of the microorganisms were undertaken.

Bract Studies:

The study of the association and occurrence of microorganisms with field bracts consisted of selecting, at random, bracts differing in age by increments of 5 days from the time they first appeared on squares and on through dehiscence of the bolls. The bracts were taken from both the Morrow and St. Joseph plots. Isolations of microorganisms were made from twenty apparently healthy field bracts collected for each age interval. To sterilize their surfaces, complete bracts were placed by individual age groups in petri dishes containing 95% ethyl alcohol for 1-2 min, and removed to petri dishes containing a solution of 1:1000 mercuric chloride for 30 min. The bracts were rinsed in sterile water for 10 min, and sections were taken aseptically from the apex, middle, and base of each bract and plated on 2% water agar, incubated at room temperature, and observed periodically. Recordings were made of microbial growth from the bract sections. Identification of most of the microorganisms recovered were made from these isolates.

Paraffin embedded, paradermal and transverse sections employing the Jensen-Sass techniques were made of bracts on bolls 5-45 days of age from the Morrow plot. Five bracts were chosen at random for the nine age lots, differing by increments of 5 days, to study the

progressive development of hyphae through the tissues. Photomicrographs, of bracts showing hyphae within them, were made.

Greenhouse Investigations

The plants were grown in bench beds at Louisiana State University Cotton Disease Laboratory using the cotton variety 'DPL 16'. The age of the bolls and bracts was determined by employing the same tagging technique used for the field grown bolls.

Boll Studies:

To study the mode of entry of *Diplodia gossypina* Cke. into cotton bolls, 4 greenhouse grown bolls for each of 12 lots, differing in age by increments of 5 days from 5-60 days, were surface inoculated with spore suspensions. To reduce drying, the plants and bolls were kept at a high moisture level by an overhead water mist for 8 daylight hours. A gentle flow of steam was introduced into the house during the night and the moisture condensed on the leaves to form dew. The inoculated bolls were removed at the end of 5 days and 2 of 4 bolls for each age group were placed in a freezer (dry) at 10° F for 36 hours. The remaining bolls of varying ages were fixed in FAA and later the pericarps were sectioned and stained using the Jensen-Sass techniques to obtain permanent slides. The frozen bolls were thawed by placing in water for 1-2 hours at 140° F. Epidermal strips were then easily removed from the exterior of the boll wall. The tissues were placed, cuticle side up, on microscope slides, stained with 1% safranin and cover slips added. Photomicrographs were taken of material which showed hyphae within or entering the bolls.

Bract Studies:

The mode of entry and colonization of bracts by D. gossypina was determined. Spore suspensions were applied only once to known age bracts as they first appeared on squares and bolls through dehiscence at 5-day intervals and maintained in the humid greenhouse. After five days, 4 inoculated bracts of each age interval were removed and surface sterilized. They were then cut into apex, middle, and base portions using aseptic methods. These sections were placed on 2% water agar and allowed to incubate at room temperature until fungal growth developed. Records were kept of the location on the bracts from which the hyphae were growing. It was assumed that the location of growth with respect to age would indicate the progression of the fungus within the bract from a given period of time.

For visual presence of the hyphae within the bracts, 4 inoculated bracts from each developmental stage at 5-day intervals were selected for paraffin embedding and paradermal and transverse sectioning to obtain prepared slides for examination. Photomicrographs were made of all slides showing fungal growth within the bracts.

EXPERIMENTAL RESULTS

Field Boll Studies:

Progressive dehiscence of field bolls was demonstrated by selecting bolls 5-45 days of age following anthesis from the Morrow, Louisiana field plot. The region of the cotton boll investigated in this research is illustrated in a free-hand section of a 20-day old boll carpel (Fig. 1).

A standard paraffin embedding and staining technique described by Jensen (10) and Sass (19) yielded cross-sectional views of the tissues of the pericarp comprising the external sutural area (Figs. 1A, 2). Continuous tissue maturation and centripetal and centrifugal suture dehiscence of field bolls was observed at 5-day intervals (Figs. 3-6).

The fundamental tissues involved in boll dehiscence were shown using a 40-day field boll suture (Fig. 2). A thick, uneven cuticle covered two to three layers of epidermal and subepidermal cells (A). The number of cell layers and wall thickness increased with age. The tissue which made up the sutural line (B) was of the typical thin-walled parenchyma type (C) which extended uninterrupted from just beneath the 2-3 layered epidermis through to the locule of the boll (I). The sutural line, which was bordered by tannin-filled parenchyma cells, extended from the epidermal cells to its encounter with developing vertical sclerenchyma fibers (F). The internal portion of the sutural line was terminated by a continuous vertical column of parenchyma tissue, the internal parenchyma ridge, which connected

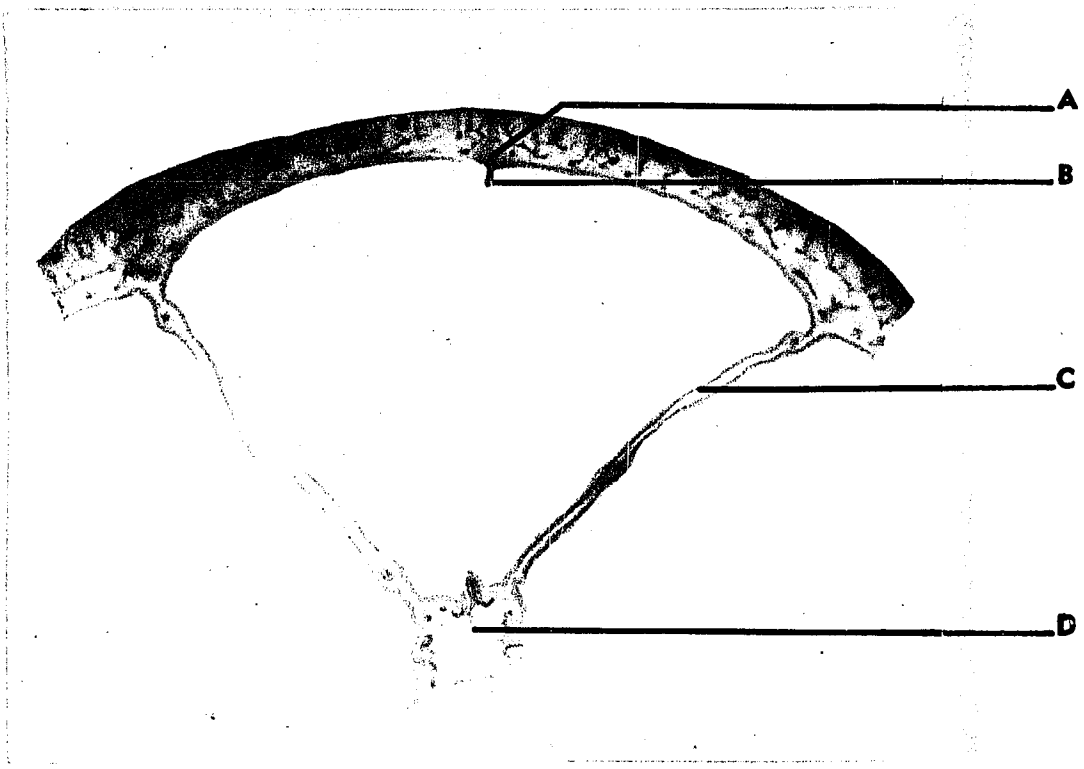
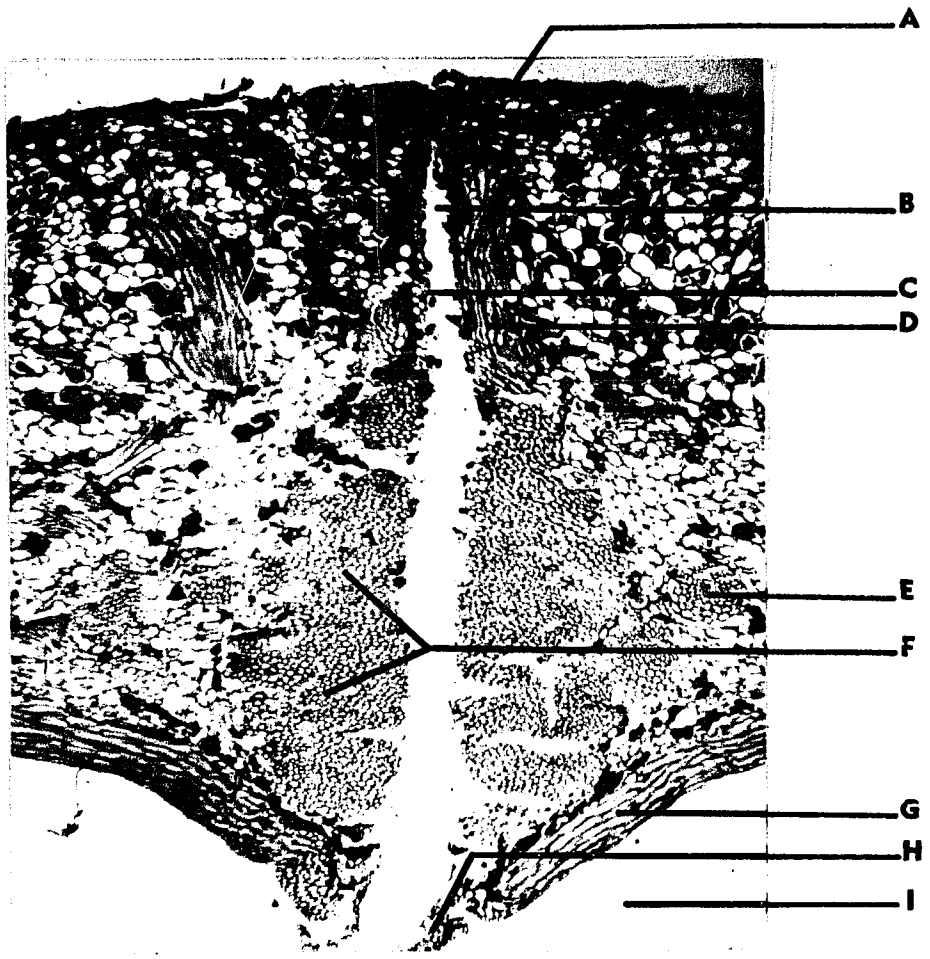


Figure 1. Transverse section of 20-day Morrow field boll carpel. (A) external suture, (B) internal parenchyma ridge, (C) endocarp, (D) internal suture. 6X



A. Epidermal layers with cuticle
B. Sutural line
C. Parenchyma tissue of suture line
D. Branching vascular ray
E. Vertical vascular tissue
F. Sclerenchyma fiber bundles
G. Sclerotized endocarp
H. Internal parenchyma ridge
I. Locule

Figure 2. Anatomy of tissues involved in centrifugal dehiscence of cotton boll capsule. 50X

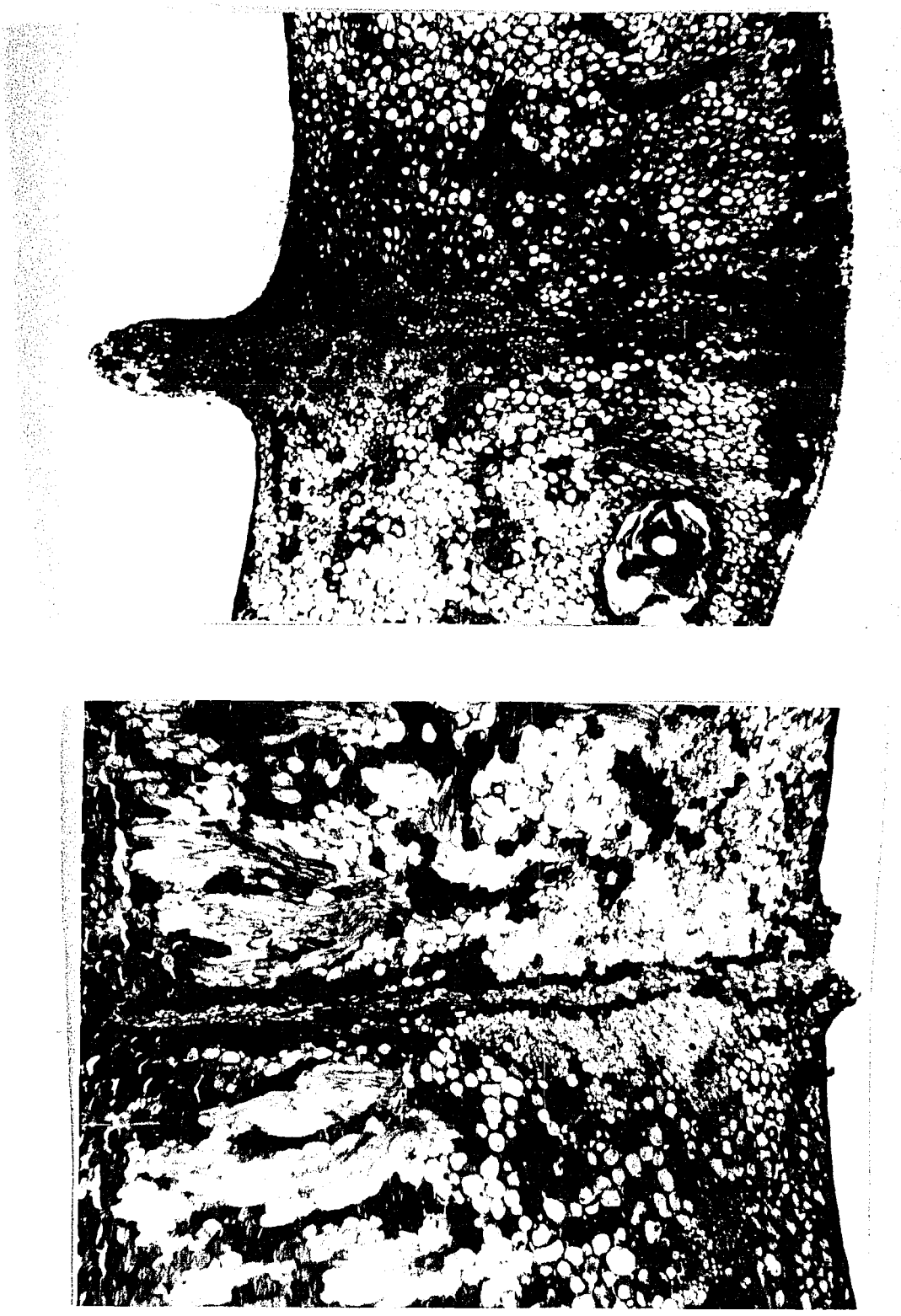


Figure 3. Progressive boll suture dehiscence of a 5-day field boll (upper, 35X) and a 10-day field boll (lower, 30X) as indicated from white blossom.

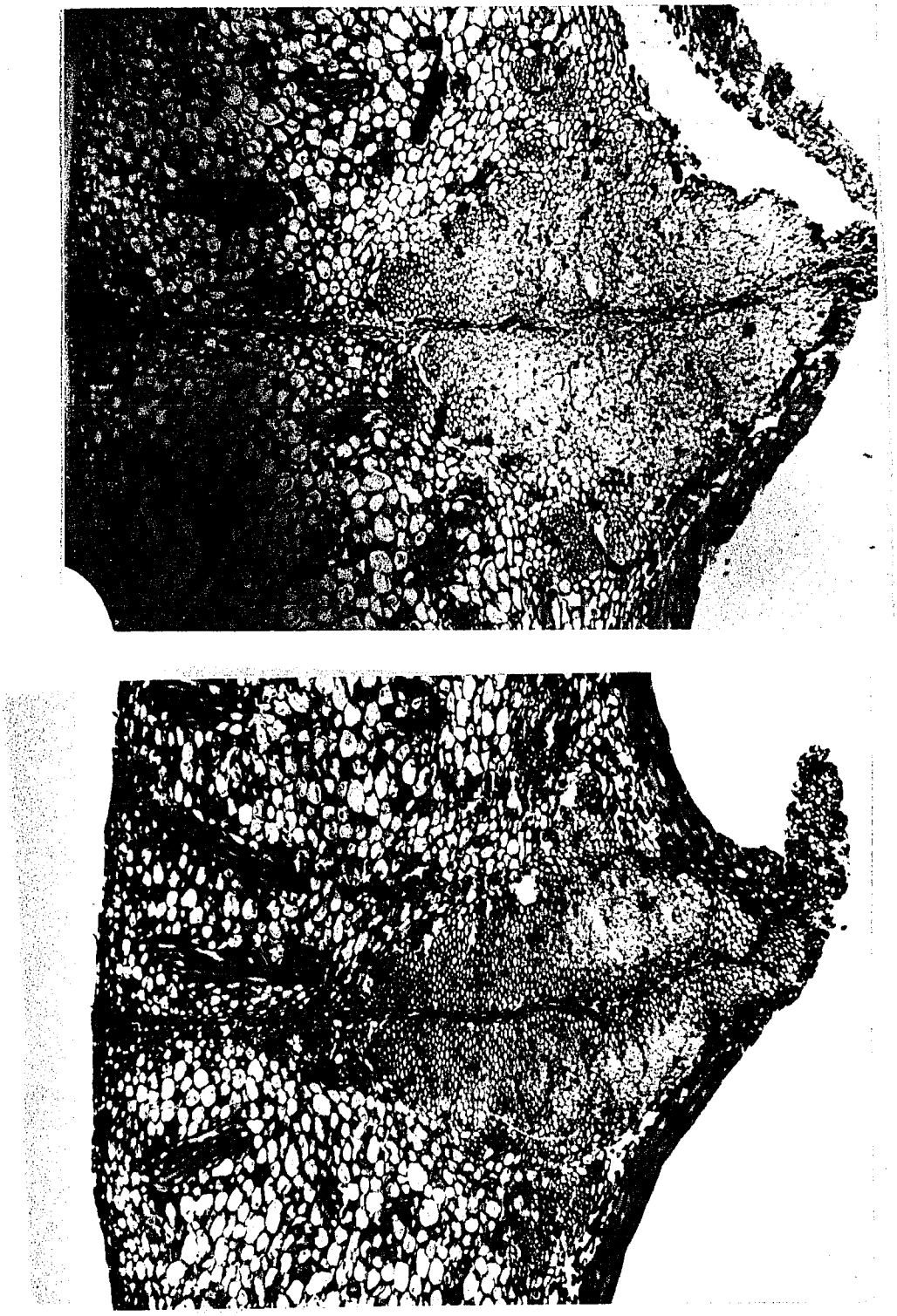


Figure 4. Progressive boll suture dehiscence of a 15-day field boll (upper, 35X) and a 20-day field boll (lower, 35X) as indicated from white blossom.

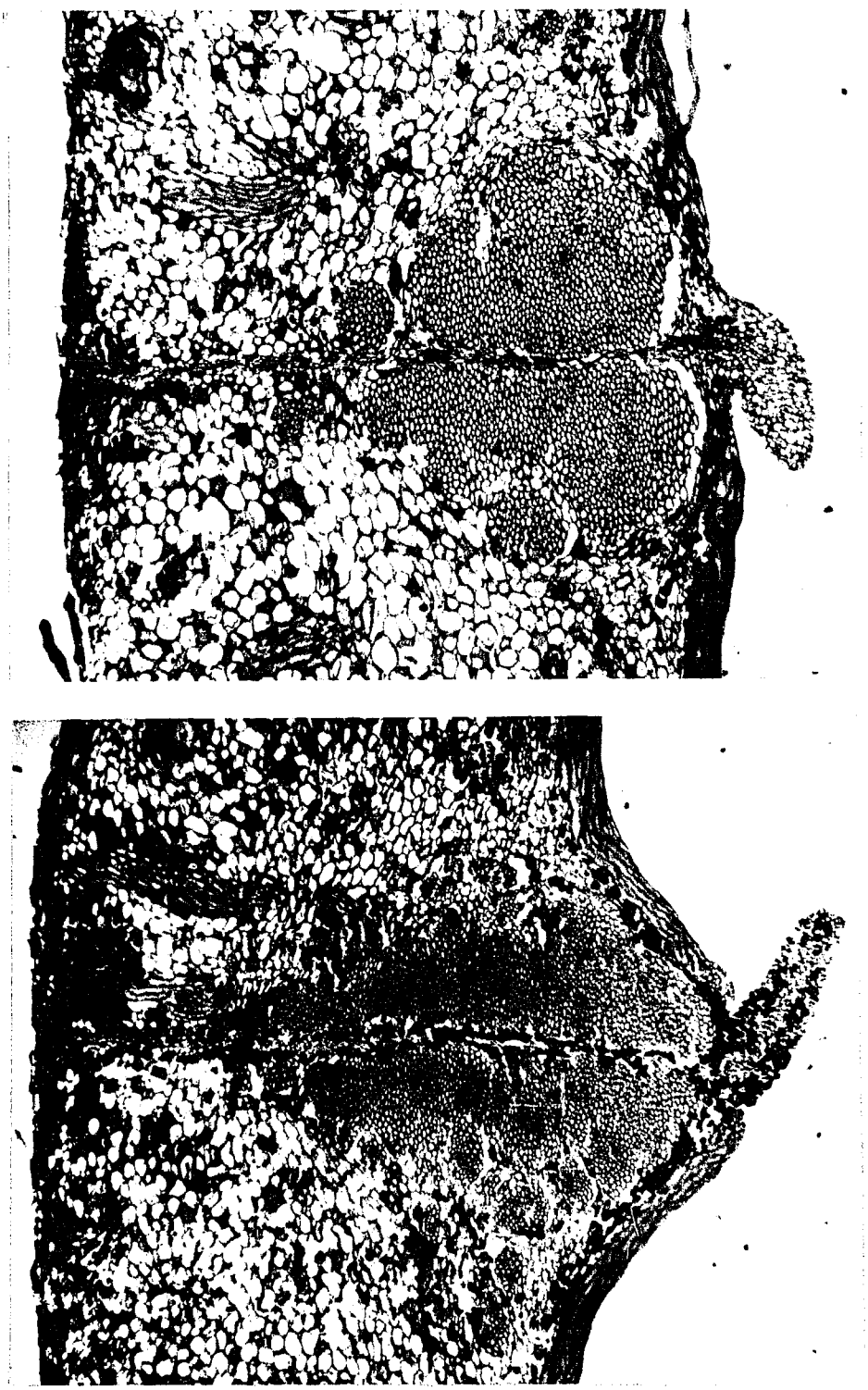


Figure 5. Progressive boll suture dehiscence of a 25-day field boll (upper, 35X) and a 30-day field boll (lower, 35X) as indicated from white blossom.

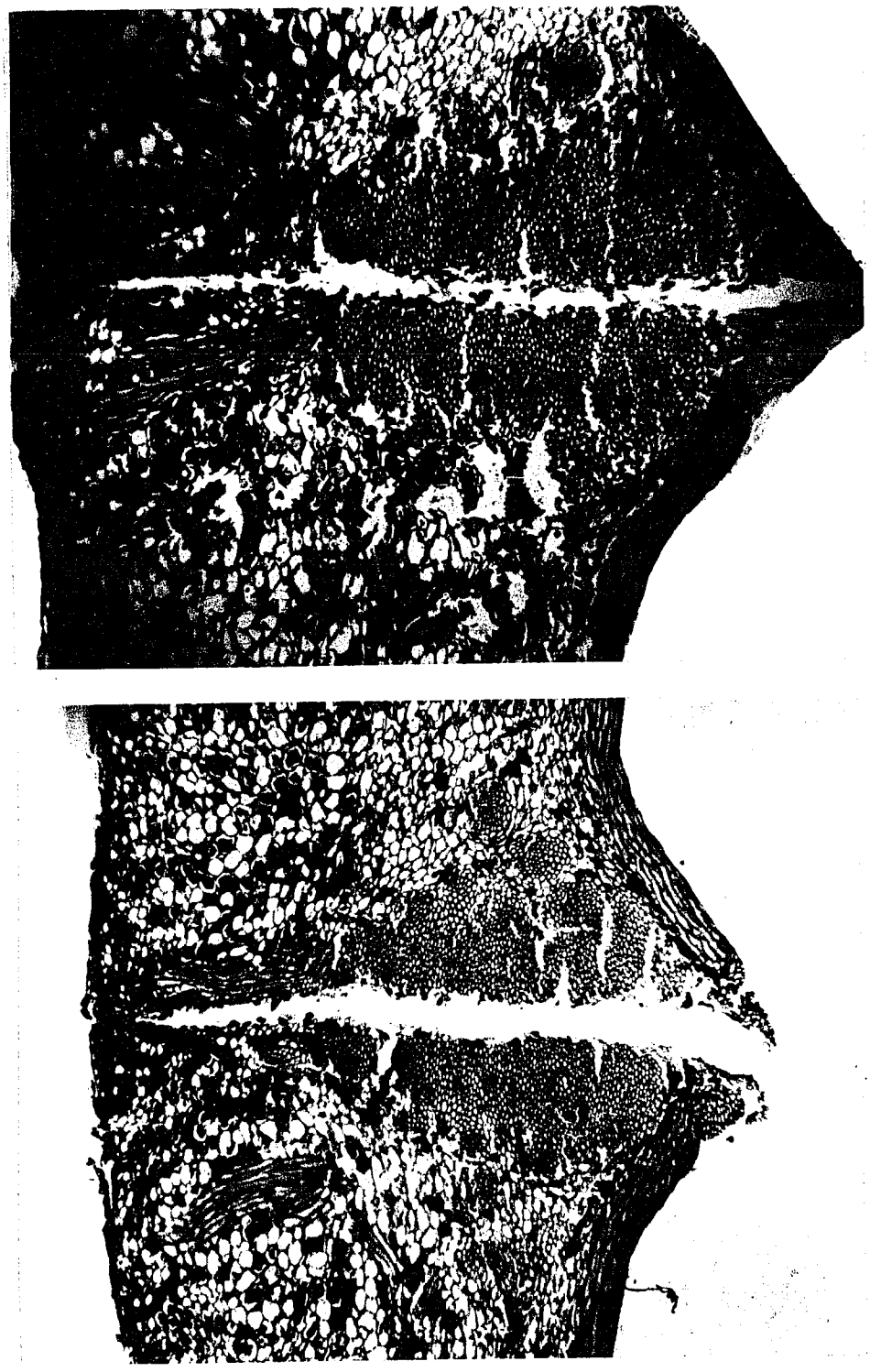


Figure 6. Progressive boll suture dehiscence of a 35-day field boll (upper, 35X) and a 40-day field boll (lower, 35X) as indicated from white blossom.

the apex of the boll with its base (Figs. 1B, 2H, 7B). The internal parenchyma ridge interrupted the almost completely solid, internal sheet of horizontal sclerenchyma fibers of the endocarp (Figs. 1C, 2G, 7). The endocarp layer lined the entire boll carpel from the external suture to the internal suture (Fig. 1) and from boll apex to base.

Developmental Boll Dehiscence:

The tissue comprising the sutural area of field bolls were observed to undergo marked morphological changes with an increase in boll age (Figs. 3-6) as described by Baranov and Maltzev (4), Gubanob (8), De Coene (7), and Joshi et al (11).

The time and point of initial sutural dehiscence of field bolls are reported herein for the first time. Dehiscence commenced from the 10th to 15th day with the progressive schizogenous separation of the sutural line parenchyma located between the sclerenchyma fiber bundles (Figs. 2C, F, 8). Sutural splitting proceeded from this point both to the internal and external limits of the pericarp (Figs. 3-6) until the 40th-45th day when dehiscence was completed.

Field Boll Fungal Penetration Studies:

A mode of entry of boll-rotting organisms into bolls in the field was determined by employing various sectioning techniques to observe fungal penetration of boll carpel wall tissues.

Paradermal sections of Morrow bolls 5-45 days of age revealed abundant mycelium covering the cuticle and/or penetrating the boll epidermal layers by way of the opened stomata (Figs. 9-12). Most of

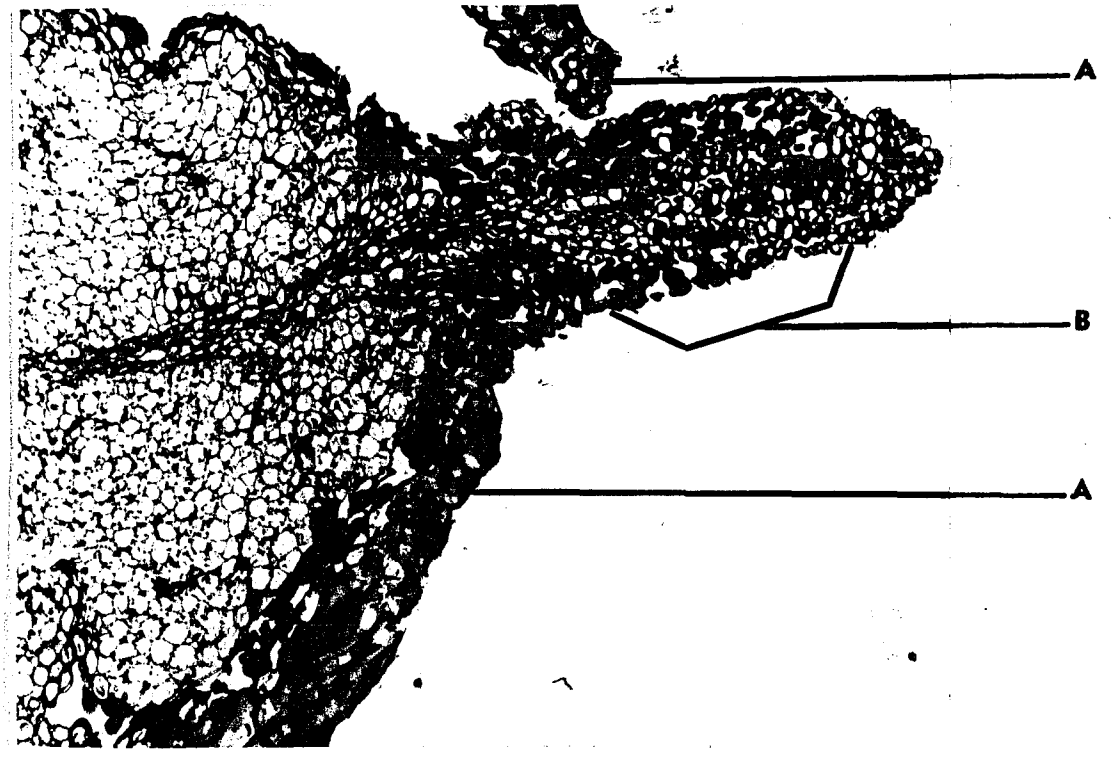


Figure 7. Termination of the sclerotized endocarp (A) at the internal parenchyma ridge (B) of a 15-day field boll carpel. The external suture is continuous with the internal parenchyma ridge as uninterrupted parenchyma tissue through to the locule. 100X

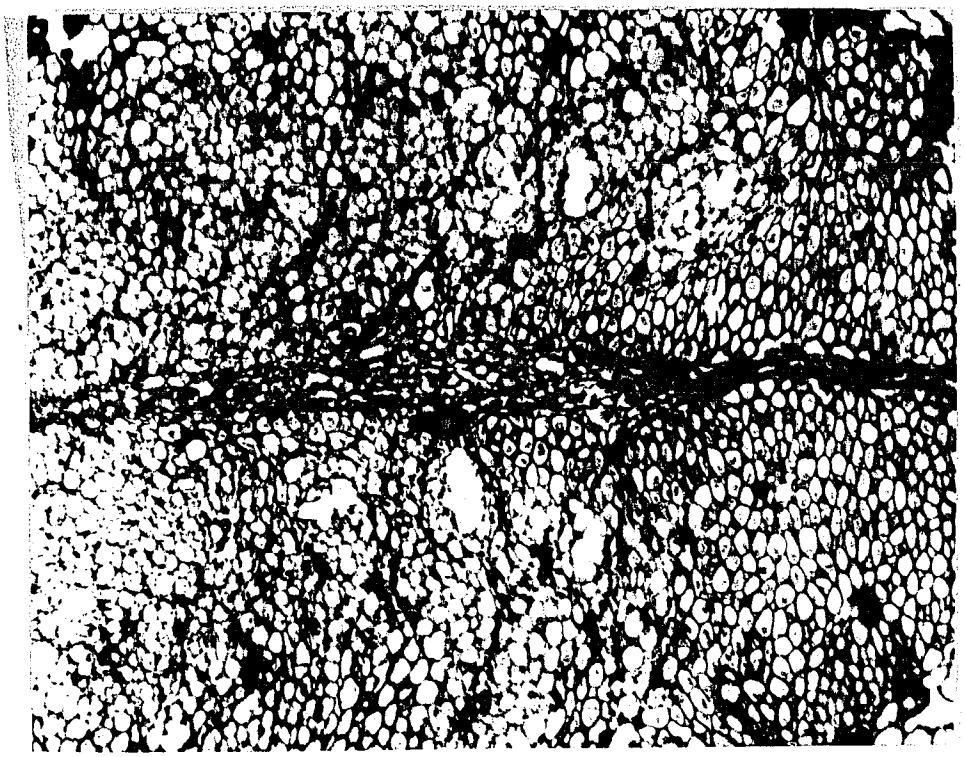


Figure 8. Fifteen day field boll suture indicating initiation of centrifugal dehiscence. Epidermis to left. 100X

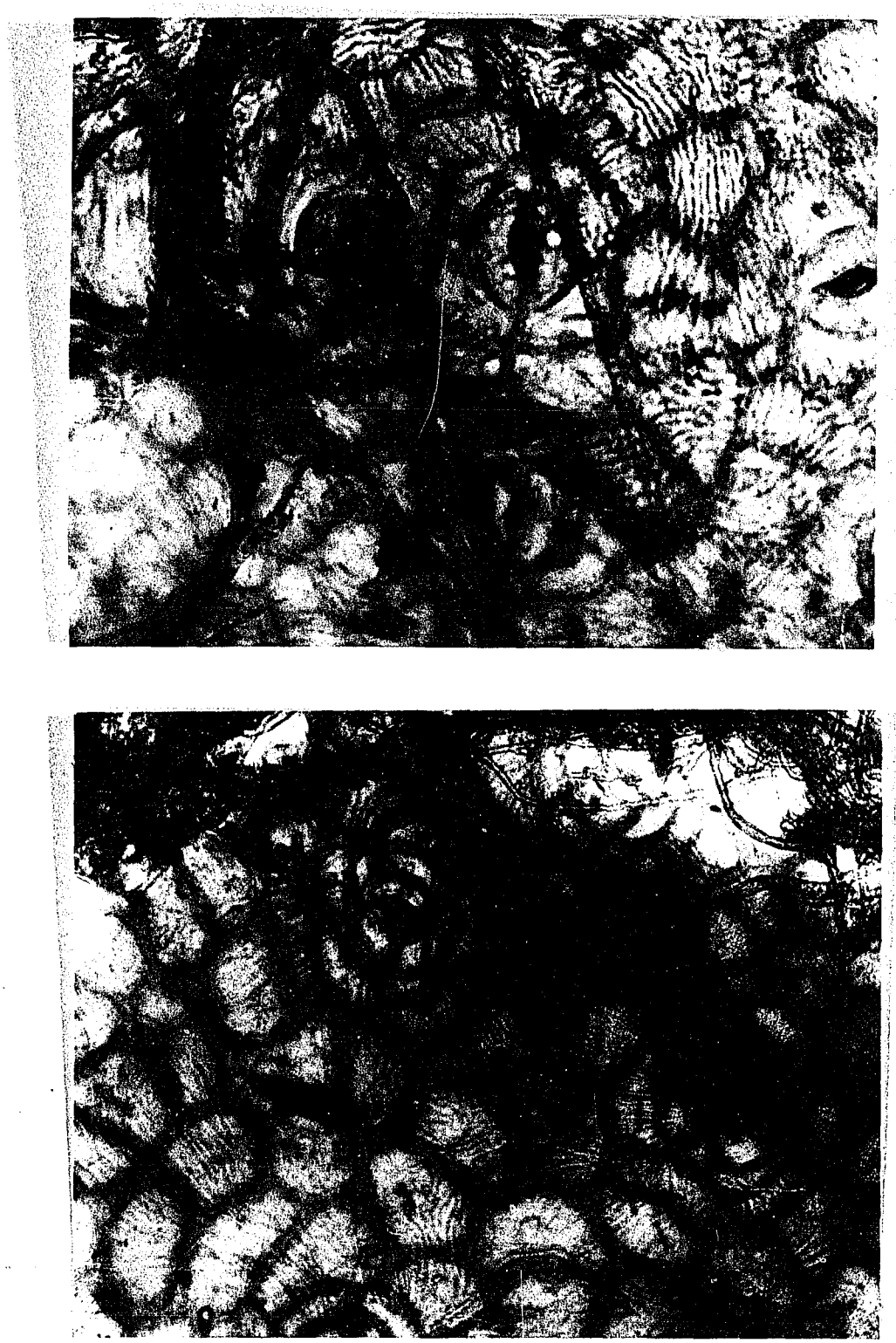


Figure 9. Paradermal sections of field bolls at 5-day intervals with mycelia randomly covering the surface of the bolls with closed stomata. Ten day field boll surface covered with Alternaria tenuis Nees mycelium (upper, 430X) and 15-day field boll surface with mycelium (lower, 430X).

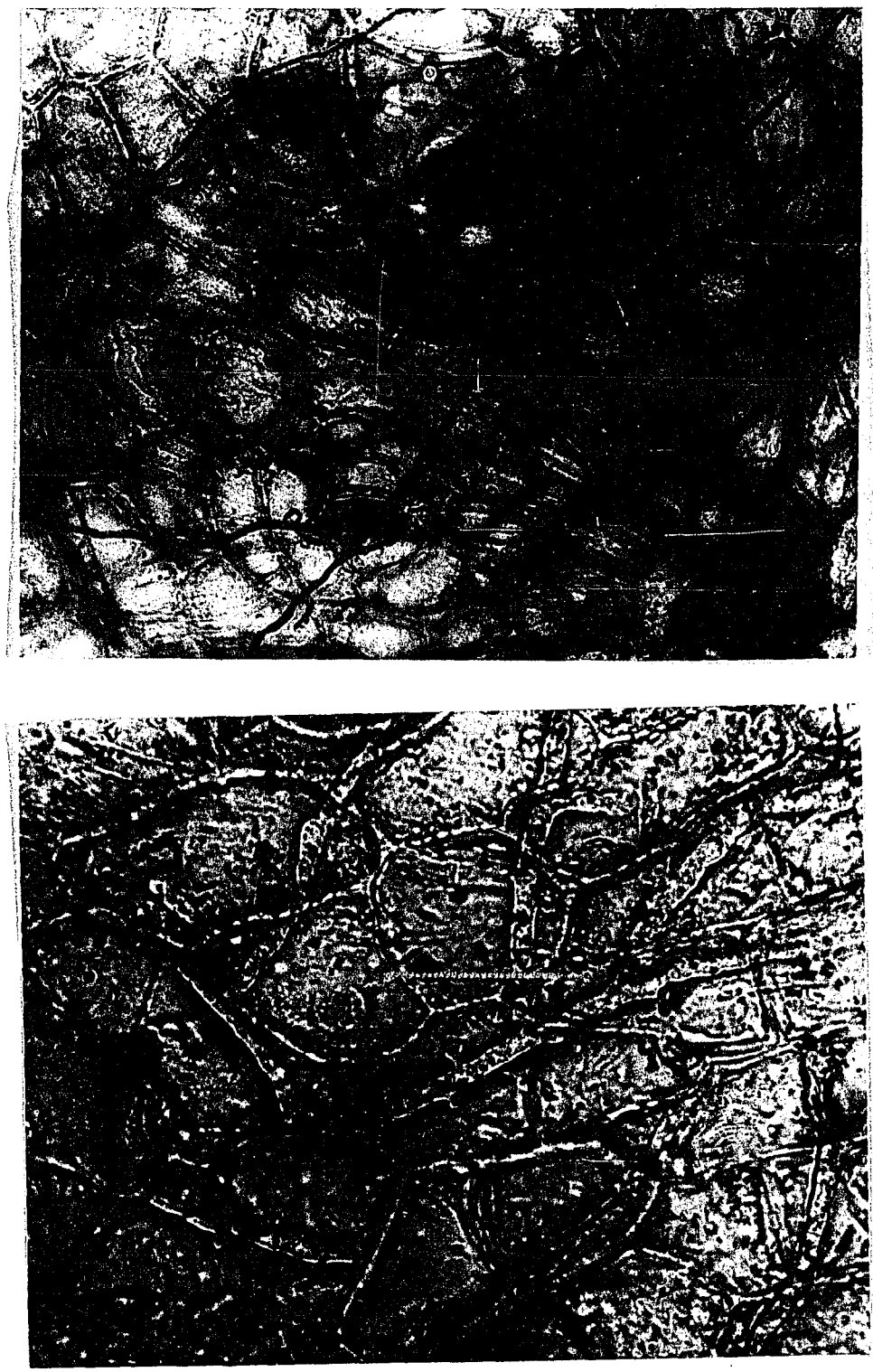


Figure 10. Paradermal sections of field bolls at 5-day intervals indicating initial fungal penetration of the carpel stomata of a 20-day field boll (upper, 430X) and fungal growth beneath the epidermis of a 25-day field boll (lower, 645X).

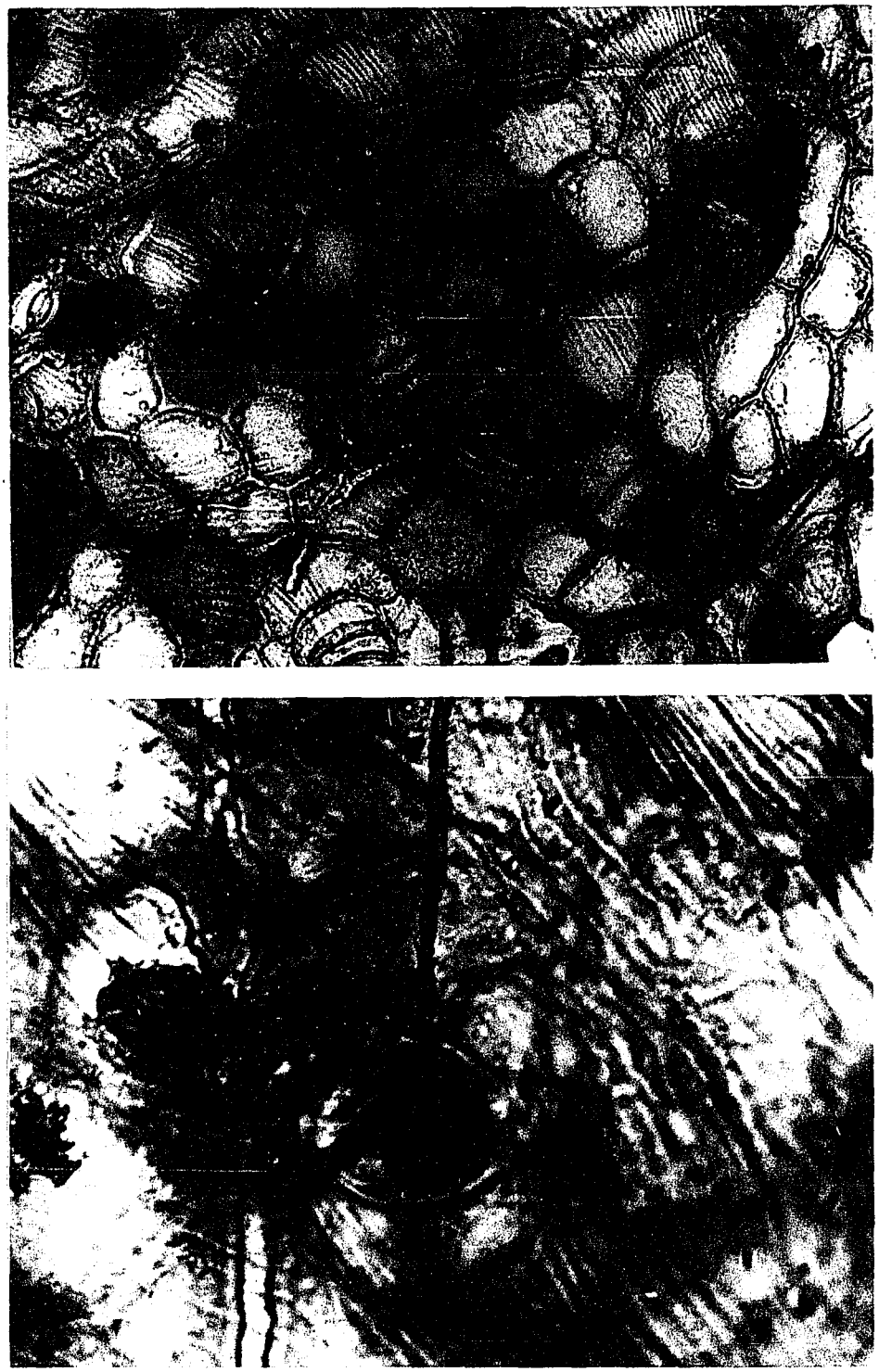


Figure 11. Paradermal sections of field bolls at 5-day intervals. Upper shows subepidermal fungal growth in a 30-day field boll (430X) and lower shows stomatal penetration and subepidermal growth of Diplodia gossypina Cke. in a 35-day field boll (600X).

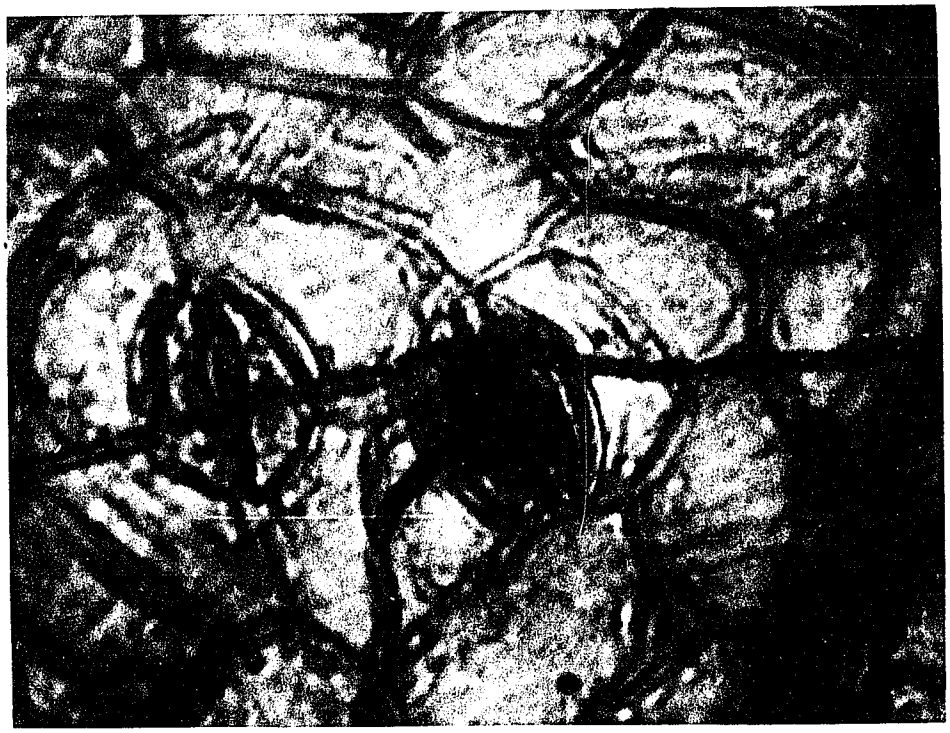


Figure 12. Paradermal section of a 40-day field boll showing stomatal penetration. 600X

the fungi, on or within the epidermal strips, were identified by placing a portion of the same epidermal peel on sterile 2% water agar for development and observation. Intercellular and intracellular hyphae were seen colonizing the epidermal layers of the boll wall (Figs. 10 lower, 11).

Guard cells of the stomata were also observed to cease functioning between the 15th and 20th day of boll development. At first only a few stomata were seen to remain open with fungal penetration following (Fig. 10 upper), then eventually most remained open as boll age increased. The cotton variety 'DPL 16' used in this study produced 15-20 stomata per microscope field when observed at 100X.

Transverse sections of bolls aged 5-45 days exhibited hyphae of various fungal species penetrating the stomata (Figs. 13, 14) and growing within the epidermal layers of the bolls (Figs. 15, 16). Microorganisms isolated and identified from the pericarps of these bolls can be seen in Table 1.

During the early stages of boll penetration, the hyphae seemed confined mainly to the first and second layers of epidermal cells (Figs. 15, 16 upper).

As the age of the boll increased, fungal growth proceeded internally to the first parenchyma cells of the mesocarp (Fig. 16 lower). Mycelial growth did not seem to continue through the remaining mesocarp tissue to the endocarp, but instead, next appeared at the sutural region above the sclerenchyma fiber bundles (Fig. 17). The hyphae appeared to be receiving nourishment from the sloughed parenchyma cells of the sutural line. Fungal activity was confined to the

Figure 13. Transverse section of 40-day field boll epidermal tissue with fungal penetration of stoma. Cuticle and first layer of epidermal cells separated from remaining boll wall tissue. 430X

Figure 14. Transverse section of 30-day field boll epidermal tissue with fungus penetrating stoma. 600X

Figure 15. Transverse sections of field boll epidermal tissue indicating fungal growth beneath the epidermis. Upper 30-day field boll infected with Alternaria tenuis Nees and lower 35-day field boll. 430X

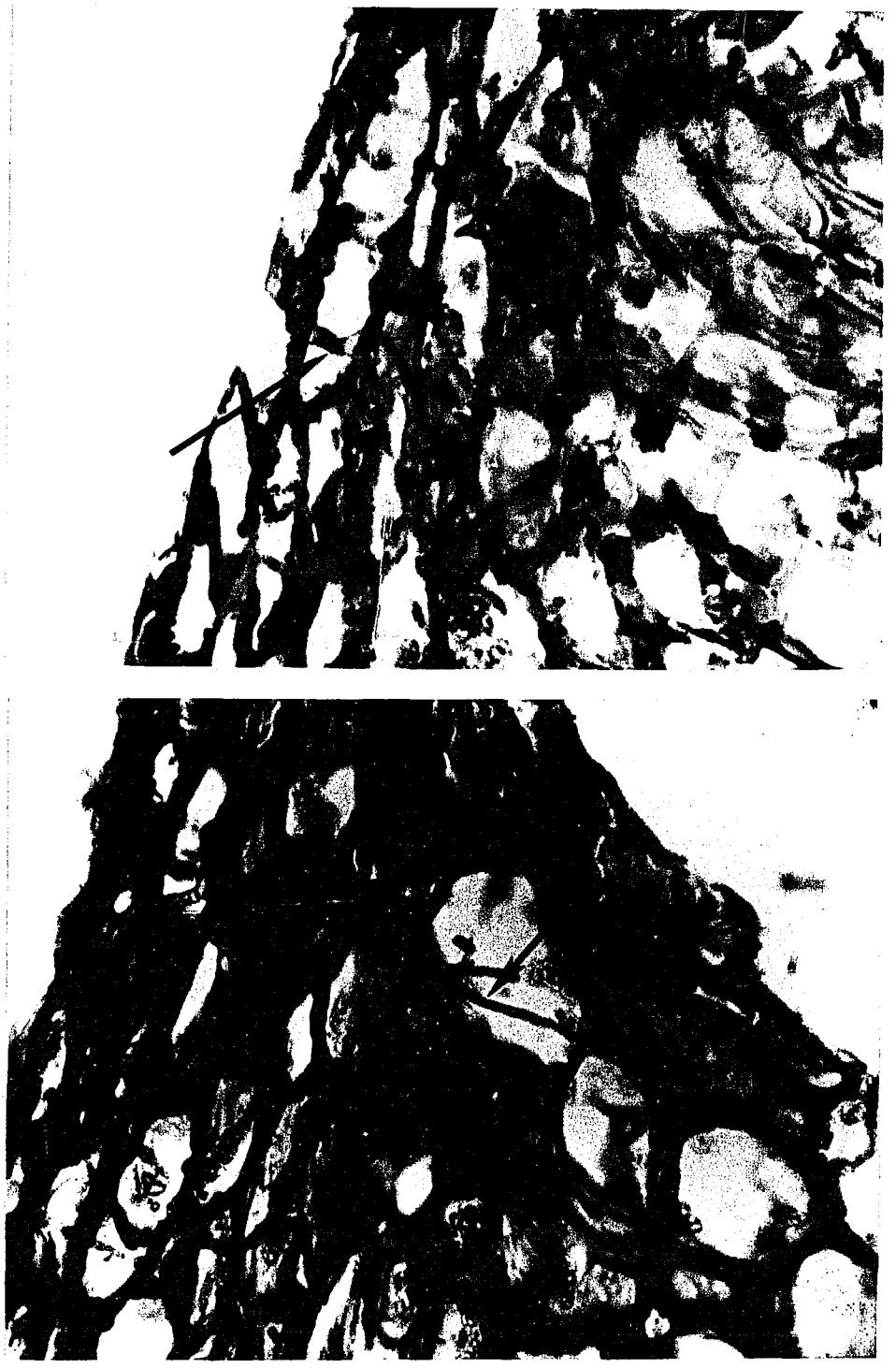


Figure 16. Transverse sections of field boll epidermal tissue indicating fungal growth beneath the epidermis. Upper 35-day field boll and lower Diplodia infected 40-day field boll. 430X

Table 1. Microorganisms isolated from internal tissues of the pericarps of field grown bolls of stated ages. /1

Microorganisms isolated from boll pericarps	Age interval (days) of field bolls /2								
	5	10	15	20	25	30	35	40	45
Fusarium sp.				+/3			+	+	+
Diplodia gossypina Cke.						+	+		+
Flavobacterium sp.				+		+		+	+
Unidentified bacteria					+	+	+		+
Alternaria tenuis Nees				+		+	+	+	
Cladosporium herbarum (Pers.) Lk.					+		+		

/1 Bolls collected from Morrow, Louisiana.
/2 Ten apparently healthy field bolls selected for each age interval.
/3 Indicates occurrence of the organism within the pericarp tissue.

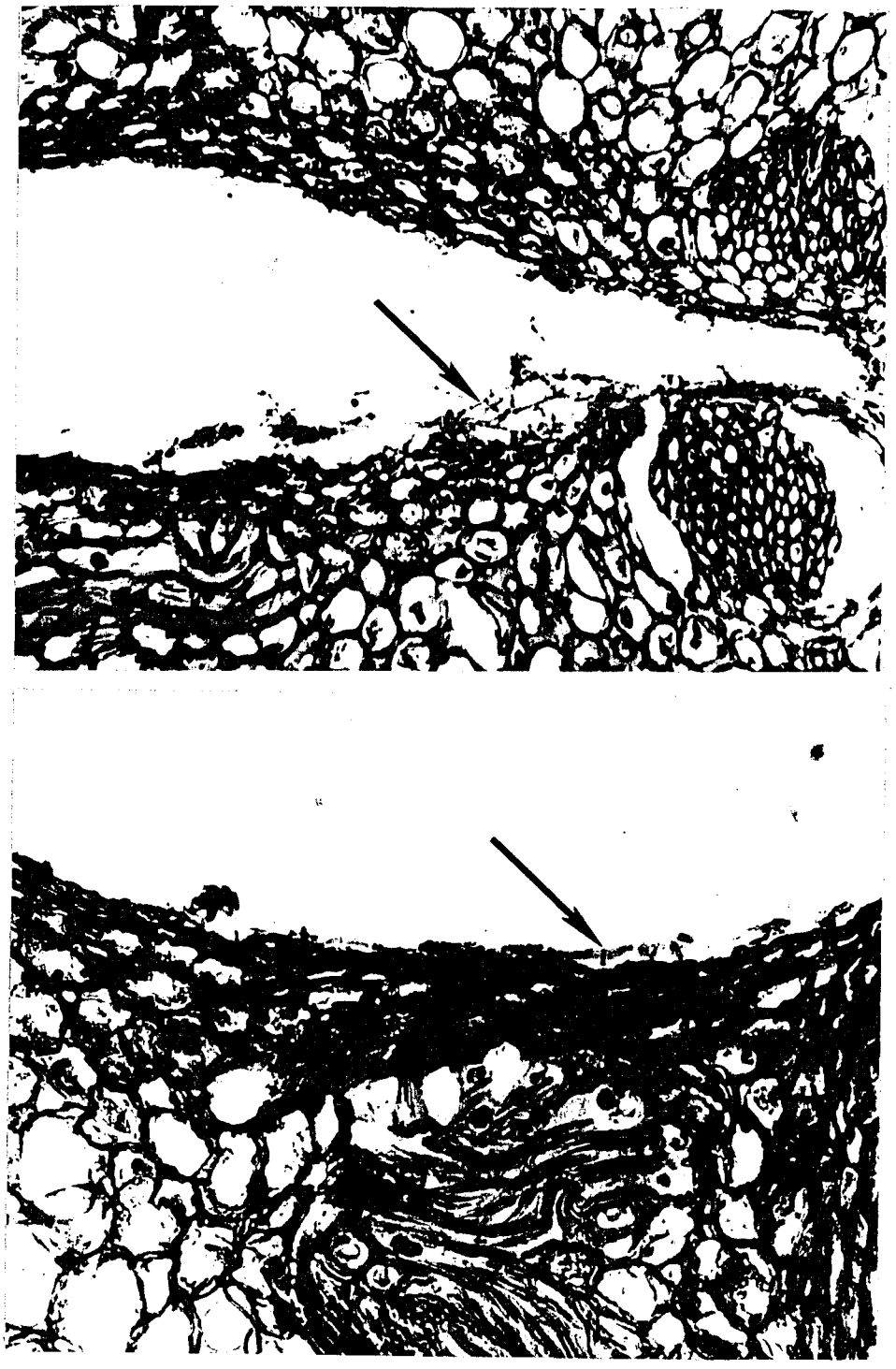


Figure 17. Transverse sections of dehiscing field boll sutures with mycelia receiving nourishment from the sloughing parenchyma cells which comprise the sutural area. Upper 40-day field boll infected with Fusarium fungus within suture and lower 35-day field boll infected with Diplodia fungus within suture. 210X

sutural parenchyma, for the most part, until dehiscence of the boll was almost complete.

Locule invasion by the different fungal species was seen to vary as to the level of pathogenicity which the fungus possessed. Diplodia gossypina Cke. exhibited a remarkable ability to invade the locule and rot the fiber before the suture had completely dehisced externally. Boll fiber infected with Fusarium sp. began to rot as the boll dehisced; however, Alternaria tenuis Nees was able to rot the fiber only after the boll was completely dehisced, exposing the fiber within.

Weather Effects on Boll Microorganisms:

Inoculum potential in the Morrow field was quite high in the early part of boll development (July 15-August 1, 1969). This could be seen as many lower bolls on the plants became rotted because of Diplodia and Fusarium infection and also the isolation of numerous species of other secondary invaders from various aged bracts (Tables 4 and 5).

The high level of inoculum was primarily caused by sufficient rainfall, overnight dew formation on the plants and adequate susceptible plant tissue, such as bracts, leaves, and lower bolls. As most of the bolls began to mature however, climatic conditions changed, resulting in a drier environment. Boll tissues matured quite rapidly as evidenced by the majority of the field bolls dehiscing between 40-45 days (Fig. 6).

The combination of low relative humidity with high temperatures during the period of major boll setting and maturation caused a

reduction of the secondary fungal inoculum available for boll rot. A complete absence of primary boll invaders, such as Colletotrichum gossypii Southworth, Pellicularia filamentosa (Pat.) Rogers, Phytophthora parasitica Dast. and others, was noted. All of the fungal isolations and identifications in this entire study failed to yield a primary boll invader, which is to be expected in a dry environment.

Boll Rot Potential Study:

Latent boll deterioration has been shown to occur by other workers (1, 2, 5, 17, 18). Various modes of penetration of the boll were attributed to microorganisms entering through: boll apex, leak in boll suture, boll base at the receptacle, nectaries, peduncle, insect or mechanical injury, direct penetration of boll wall, receptacle to placenta, and others.

In this study, the primary mode of entry into cotton bolls was seen to be through boll stomata at some period between the 15th and 20th day of boll development from white blossom. The confinement of the hyphae to the epidermal area and its delayed development in the pericarp pending further boll maturity was considered as latent infection.

To verify these observations, 10 apparently healthy bolls for each 5-day interval of age between 5-50 days were selected at random from both the Morrow and St. Joseph plots. After removal of bracts, sepals, and peduncles, the bolls were surface sterilized by dipping in 95% ethyl alcohol then soaked in 10% Clorox for 15 min. The bolls were rinsed in sterile water and placed in sterile specimen bottles

for observation. A similar lot of bolls were selected for a control or a non-treated comparison. Both the sterile and non-sterile groups were held at room temperature for 75 days.

The occurrence of microbial growth from the bolls was recorded (Figs. 18 and 19). Isolations from both the sterile and non-sterile bolls were made and the microorganisms identified in Tables 2 and 3.

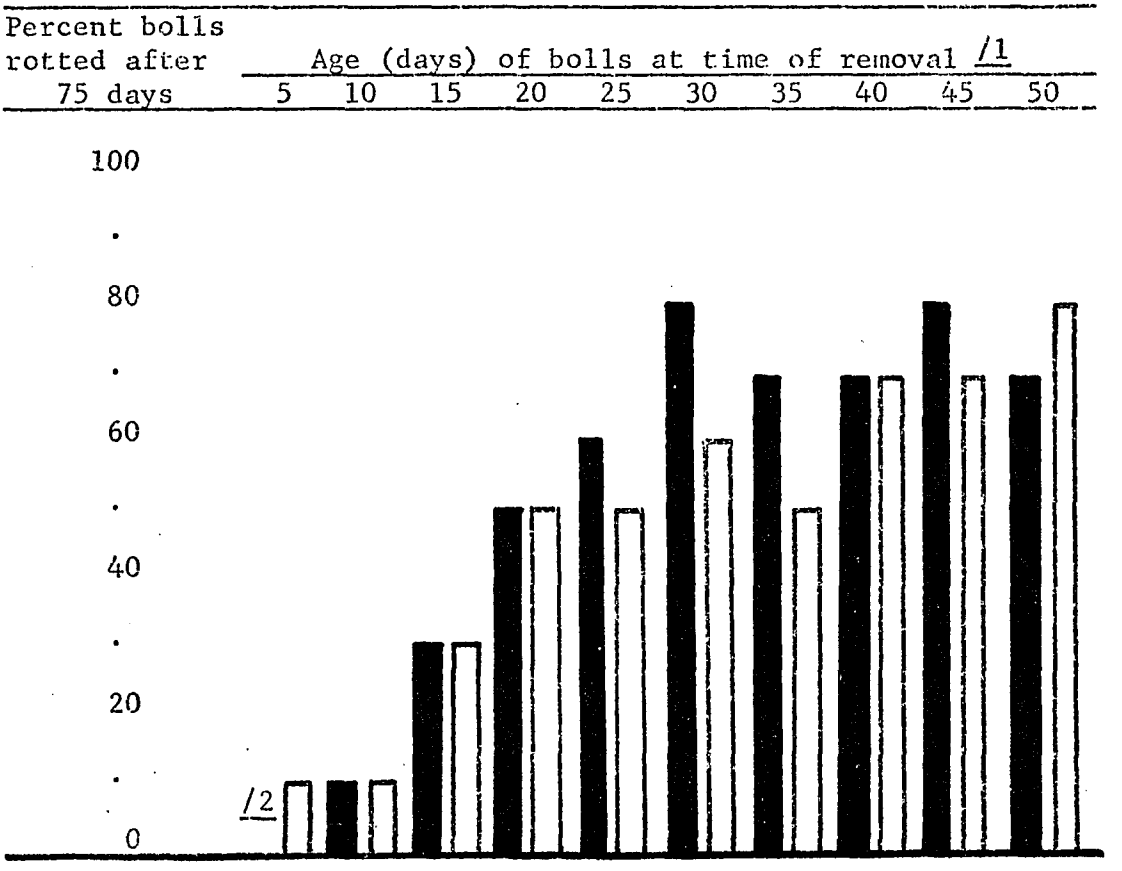
Surface sterilized bolls, which showed fungal growth in the bottles, exhibited a definite increase in rotted bolls from the 15th day of development through to the 50th day as seen in Figure 18. Bolls which were not surface sterilized showed a high percentage of rot from the 5th day of boll development (Fig. 19).

The increase in boll rot development after the 15th day for surface sterilized bolls seemed to agree with the time of boll stomatal penetration and colonization of the epidermal tissue.

Additional support to the time and mode of fungal entry of field bolls was observed when 17 Morrow and 13 St. Joseph bolls of the 5 to 20-day period developed callus tissue from the cut portion of the surface sterilized bolls (Fig. 20). Only three bolls of the treated group exhibited callus tissue formation after the 20th day.

Decrease in callus tissue production following the 20th day of boll age, appeared to be caused by the continuous maturation of the tissues comprising the bolls. Most of the tissue regions showed thickening of cell walls which usually precedes reduction of meristematic activity responsible for the proliferation of callus tissue.

Figure 18. Occurrence of rot in field grown bolls after removal from plants on stated days past white bloom and surface sterilized with 10% Clorox for 15 min.



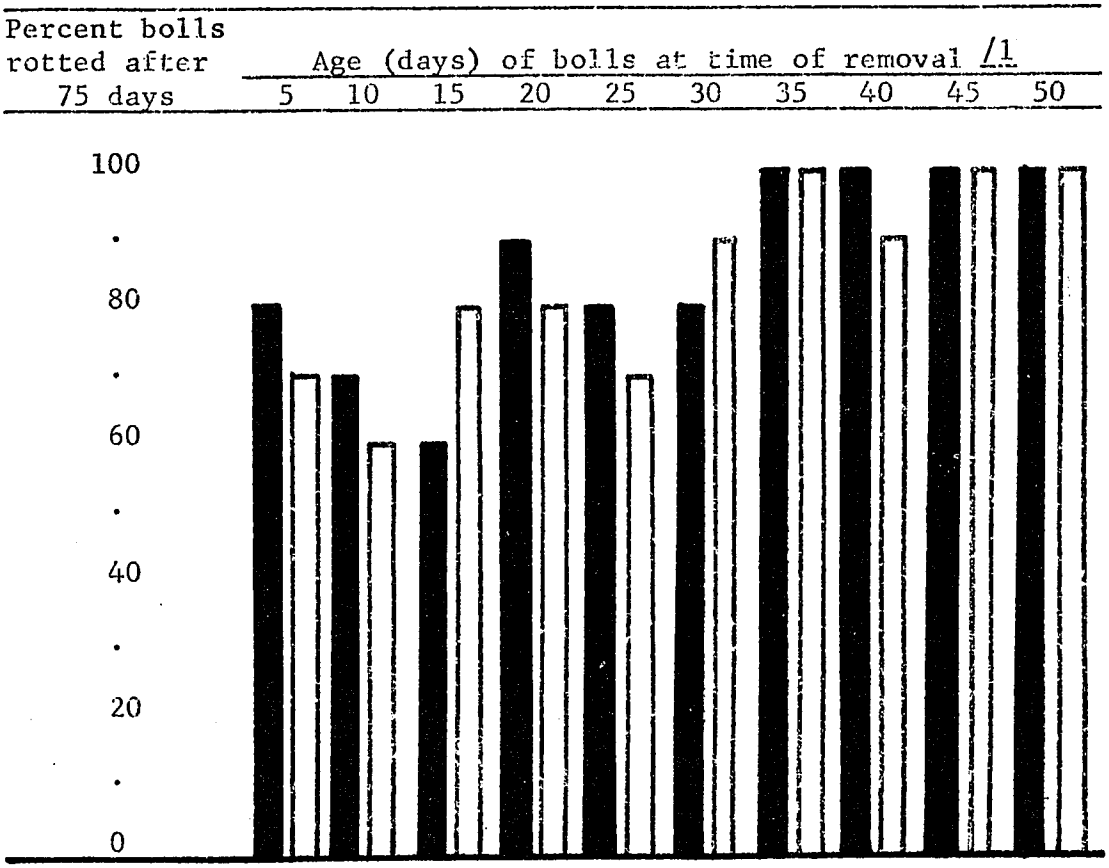
/1 Ten apparently healthy field bolls selected for each age interval.

/2 Rot possibly caused by infected minute boll weevil puncture.

■--Bolls collected from Morrow, Louisiana.

□--Bolls collected from St. Joseph, Louisiana.

Figure 19. Occurrence of rot in field grown bolls after removal from plants on stated days past white bloom and not surface sterilized.



/1 Ten apparently healthy field bolls selected for each age interval.

■--Bolls collected from Morrow, Louisiana.

□--Bolls collected from St. Joseph, Louisiana.

Table 2. Microorganisms associated with apparently healthy field bolls which were surface sterilized with 10% Clorox for 15 min and maintained in sterile, capped specimen bottles for 75 days.

Microorganisms isolated from bolls	Age interval (days) of field bolls /1									
	5	10	15	20	25	30	35	40	45	50
Fusarium sp.			M	S	MS	S	MS	M	S	MS
Alternaria tenuis Nees			M		M			S	M	MS
Chaetomium sp.								M	S	MS
Unidentified bacteria				M	MS	S	M	MS		M
Diplodia gossypina Cke.							M	MS	MS	MS
Flavobacterium sp.				M	M	S	MS			
Unidentified purple-halo fungus					M	M		M	M	
Pestalotia sp.				M						

/1 Ten apparently healthy field bolls selected from each age interval.
/M Indicates isolation from Morrow, Louisiana bolls.
/S Indicates isolation from St. Joseph, Louisiana bolls.

Table 3. Microorganisms associated with apparently healthy field bolls which were not surface sterilized but maintained in sterile, capped specimen bottles for 75 days.

Microorganisms isolated from bolls	Age interval (days) of field bolls/1									
	5	10	15	20	25	30	35	40	45	50
Fusarium sp.	MS	M	MS	MS	MS	MS	MS	MS	MS	MS
Alternaria tenuis Nees	M	M	MS	MS	MS	MS	M	MS	S	MS
Chaetomium sp.							M	MS	MS	S
Unidentified bacteria		M	MS	MS	MS	S	MS	MS		MS
Diplodia gossypina Cke.						M	M	MS	MS	MS
Flavobacterium sp.			M	M	M	MS	MS			
Paecilomyces sp.			M		MS	S		MS	MS	
Unidentified purple-halo fungus				M	M	M		M	M	
Pestalotia sp.			M	M	M					
Trichoderma sp.					M	M	MS	MS		
Curvularia sp.		M	M		MS	S	S	MS		
Helminthosporium sp.			M	M	MS	M				
Penicillium sp.				M						
Cladosporium herbarum (Pers.) Lk.				M	MS	M	M	MS		
Nigrospora sp.			M		M					
Aspergillus sp.					MS	M	MS	MS		

/1 Ten apparently healthy field bolls selected for each age interval.
/M Indicates isolation from Morrow, Louisiana bolls.
/S Indicates isolation from St. Joseph, Louisiana bolls.

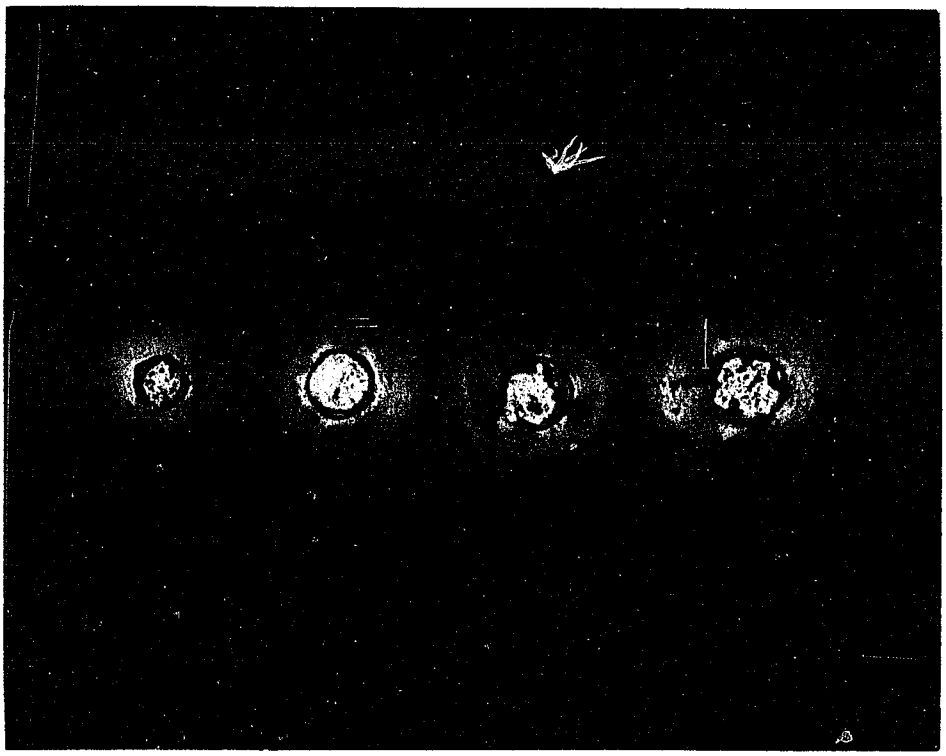


Figure 20. Typical callus tissue formed on excised bolls 5, 10, 15 and 20 days of age under aseptic conditions. Natural size

Fungal penetration of the boll stomata between the 15th and 20th days and development within the epidermal layers placed the maturing bolls in a phase of latent deterioration.

There was a much larger number of different fungi isolated from the non-sterile control bolls (Table 3) than were taken from the treated group (Table 2). This indicated the relatively weak pathogenicity of most fungi which reached the surface of a field boll and had not penetrated. These weak pathogens attacked the cotton fiber after the boll was completely dehisced.

Field Bract Studies:

The association and occurrence of microorganisms with developing field bracts was studied, and a major source of boll-rot inoculum was observed.

Age of Bracts as Related to Infection:

Morrow and St. Joseph bracts were selected at random as they first appeared on squares and throughout boll maturation to dehiscence. The time interval which separated each bract stage was 5 days. Twenty apparently healthy bracts of each age interval were collected and isolations were made.

Surface sterilization of the complete bracts in 95% ethyl alcohol for 1-2 min and then in 1:1000 mercuric chloride for 30 min was carried out. The bracts were rinsed in sterile water for 10 min, and each bract was aseptically separated into apical, middle, and basal portions. The portions were plated on 2% water agar and incubated at room temperature. Occurrence of microbial growth from within the

Table 4. Microbial growth associated with surface sterilized bracts of stated ages from white bloom indicating progressive development of microorganisms within the bracts as to time.

Age of bracts (days) at time of removal /1	Percentage of bract portions showing microbial growth					
	Apex of bract		Middle of bract		Base of bract	
1/2	65/M	45/S	50	35	30	25
5	70	65	55	40	20	30
10	55	40	50	40	25	20
15	75	60	60	45	35	25
20	60	55	55	45	25	20
(anthesis) 0	75	65	65	55	30	25
5/3	85	55	70	45	30	30
10	80	70	70	60	50	45
15	85	75	60	50	45	45
20	75	70	75	60	50	40
25	90	80	80	65	60	50
30	90	85	80	70	70	55
35	100	85	75	75	75	65
40	90	90	90	80	80	70
45	100	95	100	90	100	90
50	100	100	100	100	100	100
55	100	100	100	100	100	100

/1 Twenty whole, apparently healthy field bracts selected for each age interval.
/2 Bracts selected from squares.
/3 Bracts selected from bolls.
/M Bracts collected from Morrow, Louisiana.
/S Bracts collected from St. Joseph, Louisiana.

bract tissues was recorded as percentage of infected bract portions in Table 4.

This study established for the first time the presence of microbial activity within the very earliest formed bracts of field squares. Bract colonization by these microorganisms at an early stage in floral development allowed a continuous inoculum build-up for future boll infection throughout the boll development period.

It was noted that the predominant bract portions involved in initial microbial colonization were the bract teeth or apices, which is in agreement with Luke (12). However, Table 4 shows that further infection of the bract middle and basal tissues was detected. The lowest percentage of infection was exhibited by the bract bases.

All bract regions were infected by the 45th day following white blossom, when most of the bracts in the field plots had rotted from the bolls and most of the bolls dehisced.

Microorganisms Isolated from Field Bracts:

Identifications were made of most of the microbial isolates taken from the bract portions and recorded as a composite in Table 5 indicating their presence in the complete bracts of stated ages from both Morrow and St. Joseph plots.

The predominant bract invader was Alternaria tenuis Nees, which was isolated from bracts of every age interval (Fig. 21). The bulk of bract infection by microorganisms other than A. tenuis occurred from the 10th day following white blossom (anthesis) through to boll dehiscence.

Table 5. Occurrence of microorganisms isolated from field grown bracts of stated ages taken from Morrow and St. Joseph, Louisiana. (Apex, middle, and base)

Microorganisms isolated from bract portions	Bract ages (days) /1																			
	Square ages					Anthesis/2	Boll ages													
	1	5	10	15	20	0	5	10	15	20	25	30	35	40	45	50	55			
Diplodia gossypina Cke.										+	+	+	+	+	+	+	+			
Verticillium sp.									+	+		+		+	+	+				
Paecilomyces sp.								+	+		+	+								
Fusarium sp.			+	+	+			+	+	+		+		+	+					
Curvularia sp.								+	+		+	+		+						
Helminthosporium sp.									+	+	+			+	+					
Flavobacterium sp.					+		+		+	+	+		+	+	+					
Unidentified bacteria							+		+	+		+		+	+					
Chaetomium sp.											+			+		+	+			
Unidentified purple-halo fungus								+	+		+	+	+			+	+			
Aspergillus sp.						+		+		+	+			+						
Epicoccum sp.												+		+						
Hyaloflorae sp.										+		+	+							
Helicosporium sp.											+	+				+				
Penicillium sp.				+	+			+	+											
Pestalotia sp.						+		+	+		+									
Cladosporium herbarum (Pers.) Lk.										+	+			+	+	+				
Diplococcium sp.								+	+		+									
Monilia sp.												+	+							
Alternaria tenuis Nees	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			

/1 Twenty whole, apparently healthy field bracts selected for each age interval.

/2 Anthesis began on June 23-25, 1969.

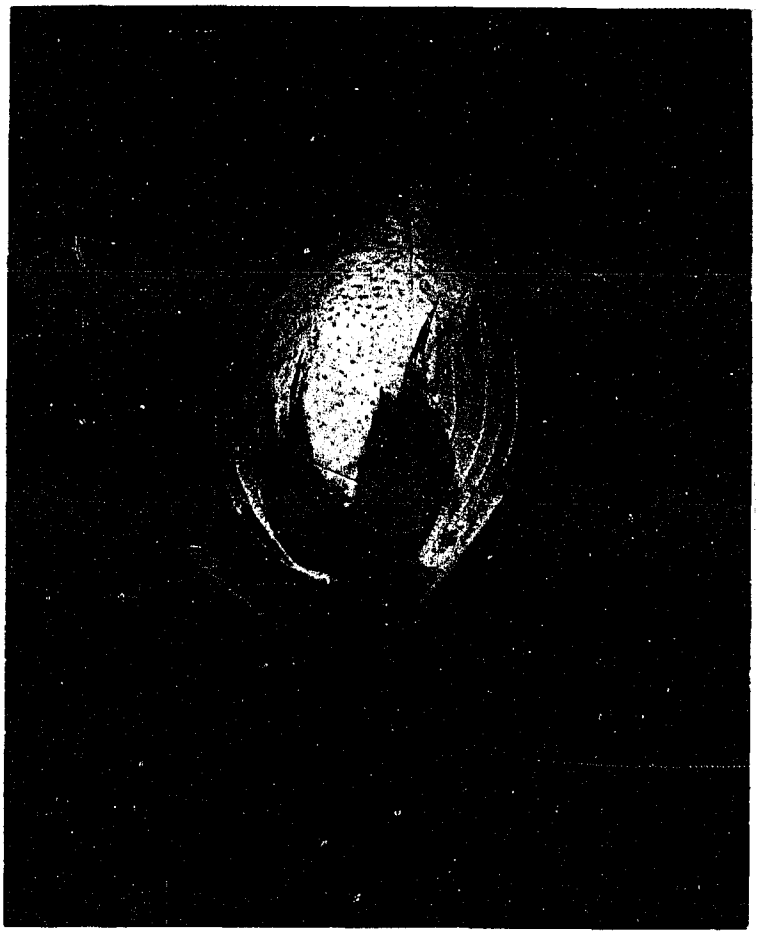


Figure 21. Forty day Morrow field boll showing typical bract decay caused by Alternaria tenuis Nees entering the bract tips and the mycelium proceeding to the base. Natural size

The microorganisms associated with field bracts could be favorably compared to those isolated from the external and internal regions of the field bolls as seen in Tables 2 and 3 of the previous study.

Visual Presence of Fungal Infection in Bracts:

Paraffin-embedded paradermal and transverse sections of Morrow field bracts, 5-45 days of age, revealed abundant hyphae progressing through the maturing bract tissues.

Initial fungal penetration of the bracts occurred near the apices through normal epidermal stomata or modified stomata--the hydathodes (Figs. 22, 41).

Following the entry into the bracts, the hyphae proceeded intercellularly through the bract mesophyll to the base (Figs. 23, 24, 25, 26).

Again, the microorganisms involved in the bract infection were of the secondary pathogen class as observed in the boll studies.

Direct parasitism of the bracts was not observed; rather, a saprophytic-type existence was noted. Bract decay caused by these microorganisms was seen only after bract senility commenced from the apex.

Once colonization and decay of the bract apex by the fungus was accomplished, the hyphae proceeded down the bract to the base as the bract age increased (Figs. 24 lower, 25 upper, 26).

Greenhouse Boll Penetration Studies:

Cotton variety 'DPL 16' plants were grown in bench beds at the Louisiana State University Cotton Disease Laboratory to study the mode of penetration of an artificially inoculated fungus into boll surfaces.

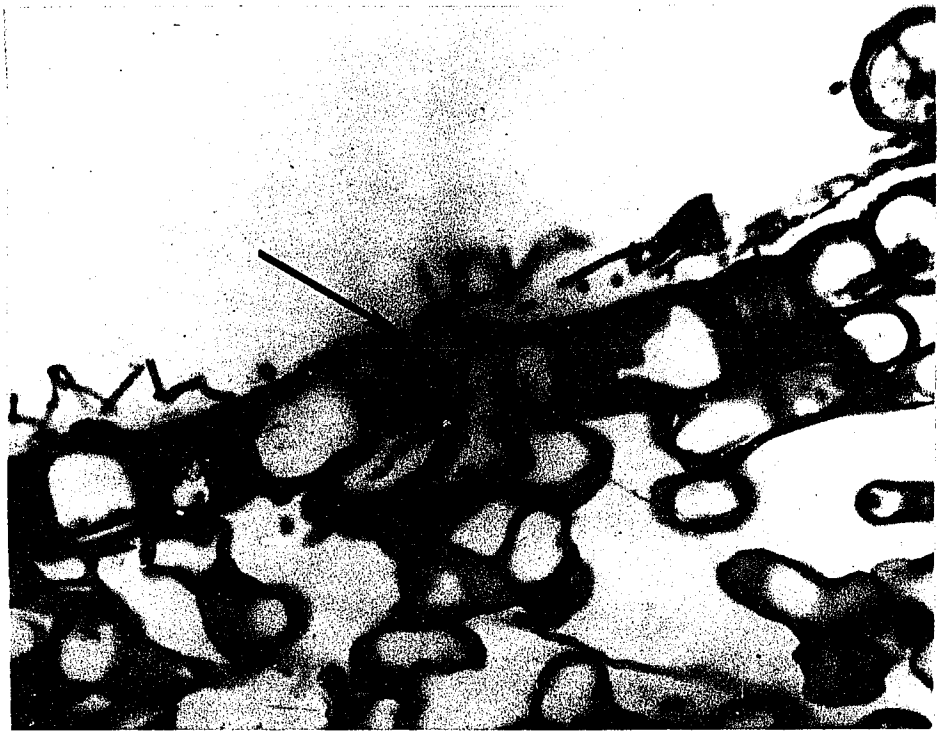


Figure 22. Transverse section of a 30-day field bract near the apex showing stomatal penetration by Alternaria tenuis Nees. 430X

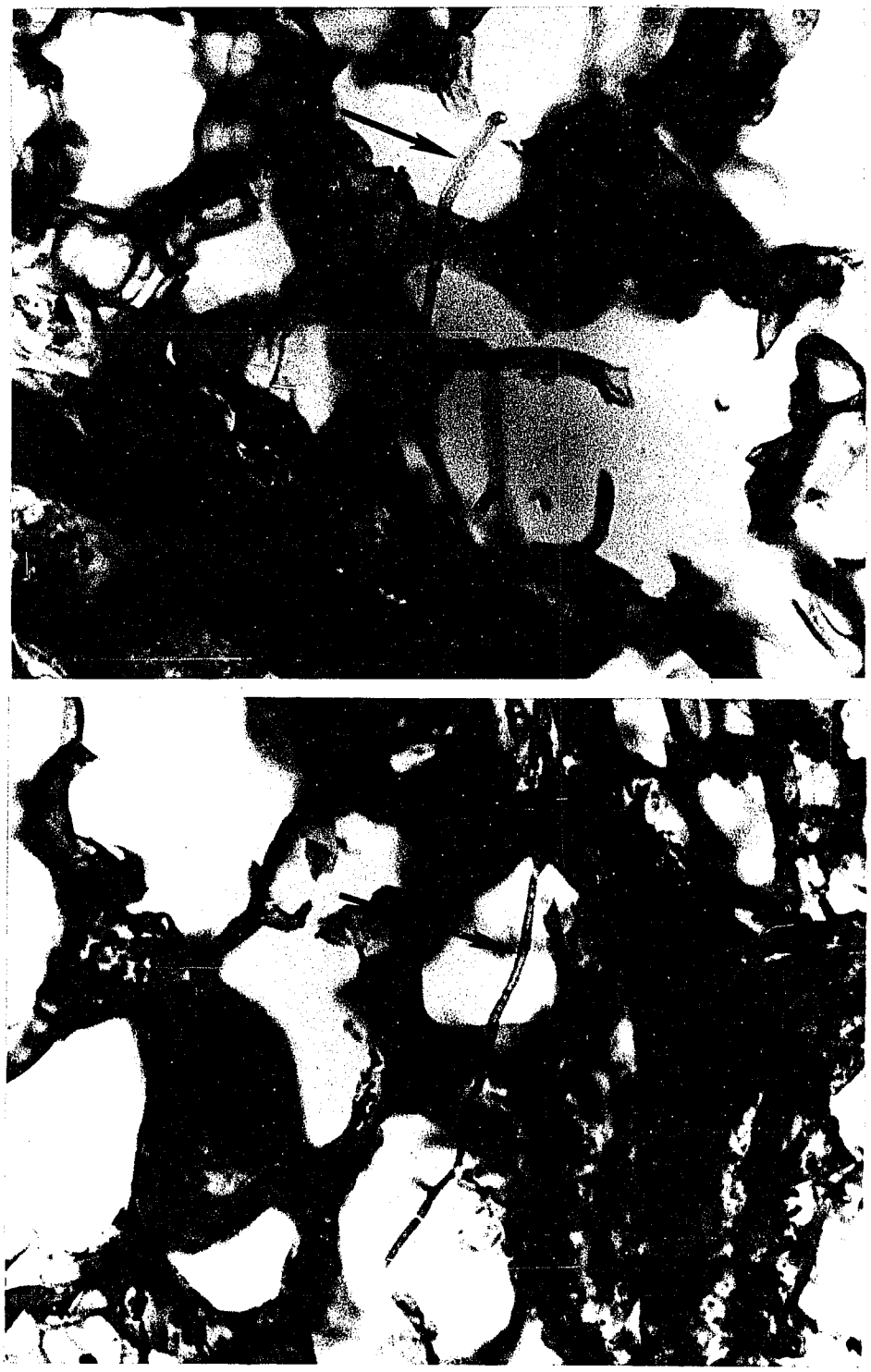


Figure 23. Paradermal sections of field bracts with intercellular hyphae. Upper 10-day bract apex and lower 20-day bract apex infected with Alternaria tenuis Nees. 645X

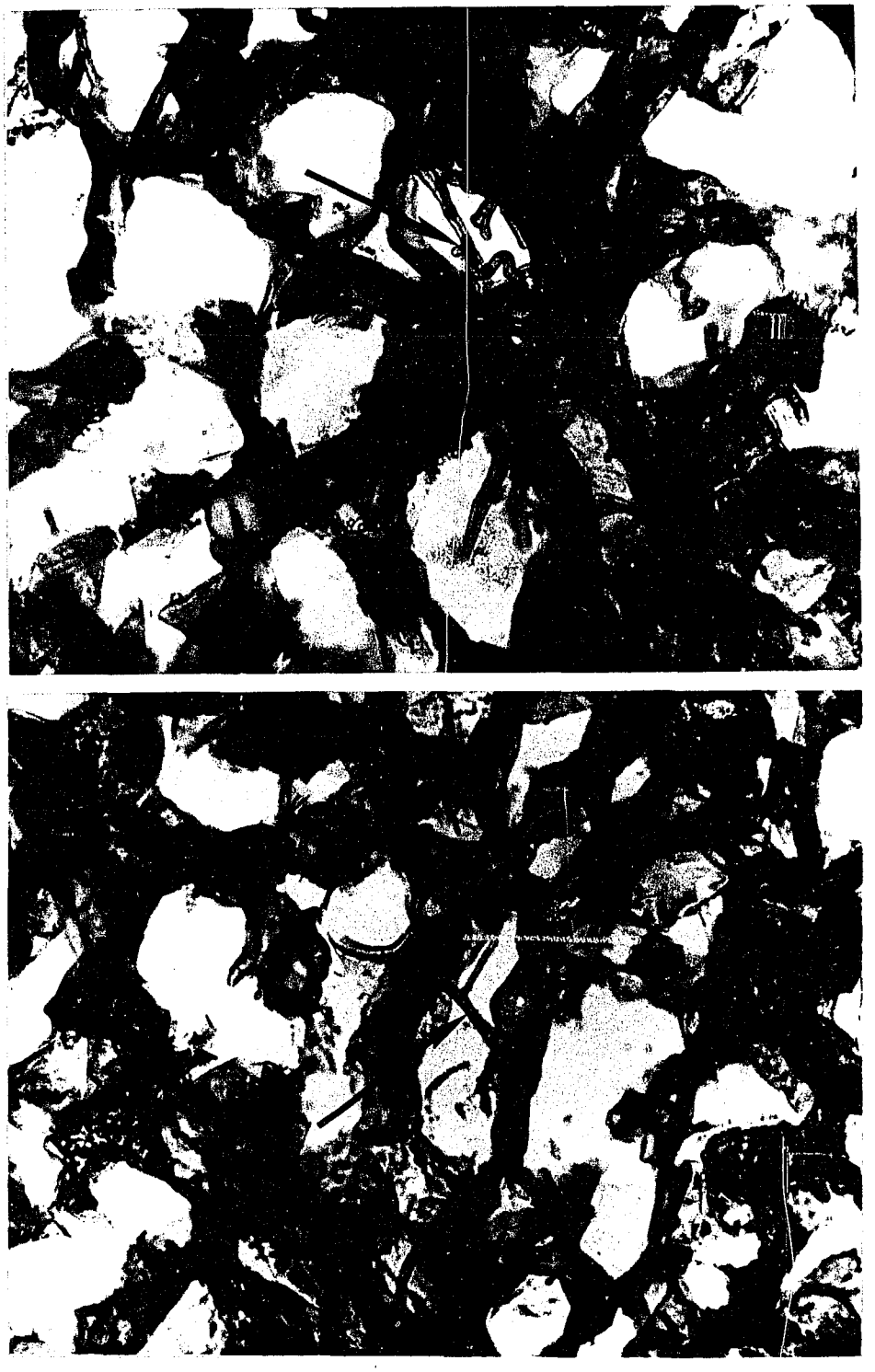


Figure 24. Paradermal sections of field bracts with intercellular hyphae. Upper 30-day bract apex infected with Diplodia gossypina Cke. and lower 30-day bract middle. 645X

Figure 25. Paradermal sections of field bracts with intercellular hyphae. Upper 30-day bract base and lower 40-day bract apex infected with Alternaria tenuis Nees. 750X

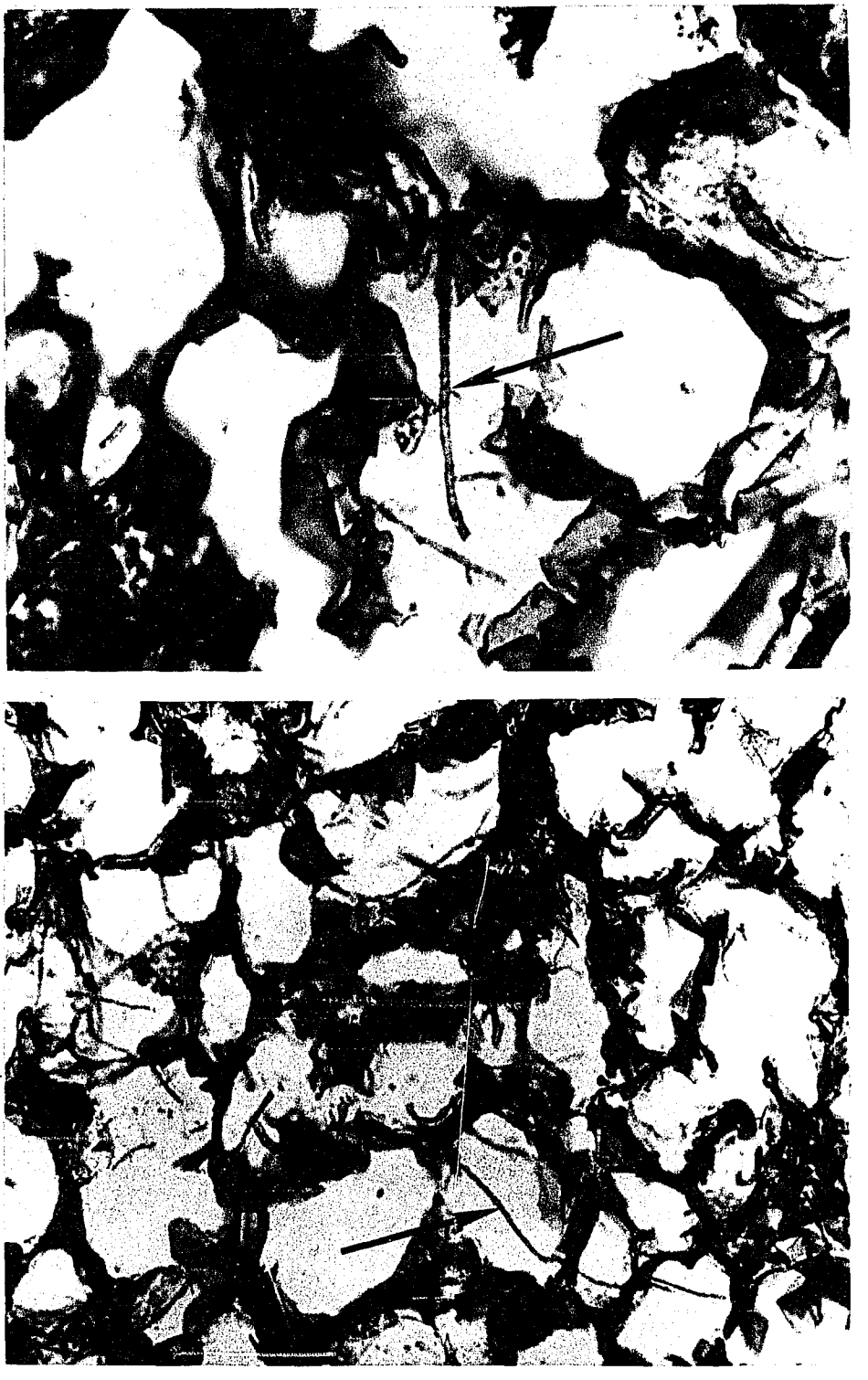


Figure 26. Paradermal sections of field bracts with intercellular hyphae. Upper 40-day bract middle infected with Alternaria tenuis Nees (750X) and lower 40-day bract base infected with Fusarium sp. (645X).

Four greenhouse bolls of each age (5-60 days of 5-day intervals) were surface inoculated with spore suspensions of Diplodia gossypina Cke. This particular culture of D. gossypina was taken from a field boll isolate of the previous study so that its pathogenicity was known.

Constant high humidity was maintained daily for 8 daylight hours by an overhead water mister. A gentle flow of steam during the night encouraged dew formation on the leaves and bolls.

The inoculated bolls were removed at the end of 5 days. Two of the four bolls for each age interval were placed in a freezer at 10° F for 36 hours. The remaining bolls of varying ages were prepared for paraffin embedding, transverse sectioning, and staining.

The frozen bolls were thawed in 140° F water for 1-2 hours. The cuticle and first and second epidermal layers were easily peeled from the remaining boll tissue. These sections were placed cuticle side up on microscope slides, stained with safranin, and cover slips were added.

Fungal growth was observed on practically all of the boll sections made. D. gossypina spores had germinated on and mycelium had covered the surface of the variously aged bolls (Figs. 27, 28, 29).

The optimum conditions of more than adequate moisture, fertilizer and the "greenhouse effect" produced more succulent plants and later maturing bolls than those found in the field studies. Evidence of this could be seen from the age at which guard cells of the bolls began to cease functioning (between 35 and 40 days), and the time of stomatal penetration by the fungus, approximately 40 days (Fig. 28 lower).

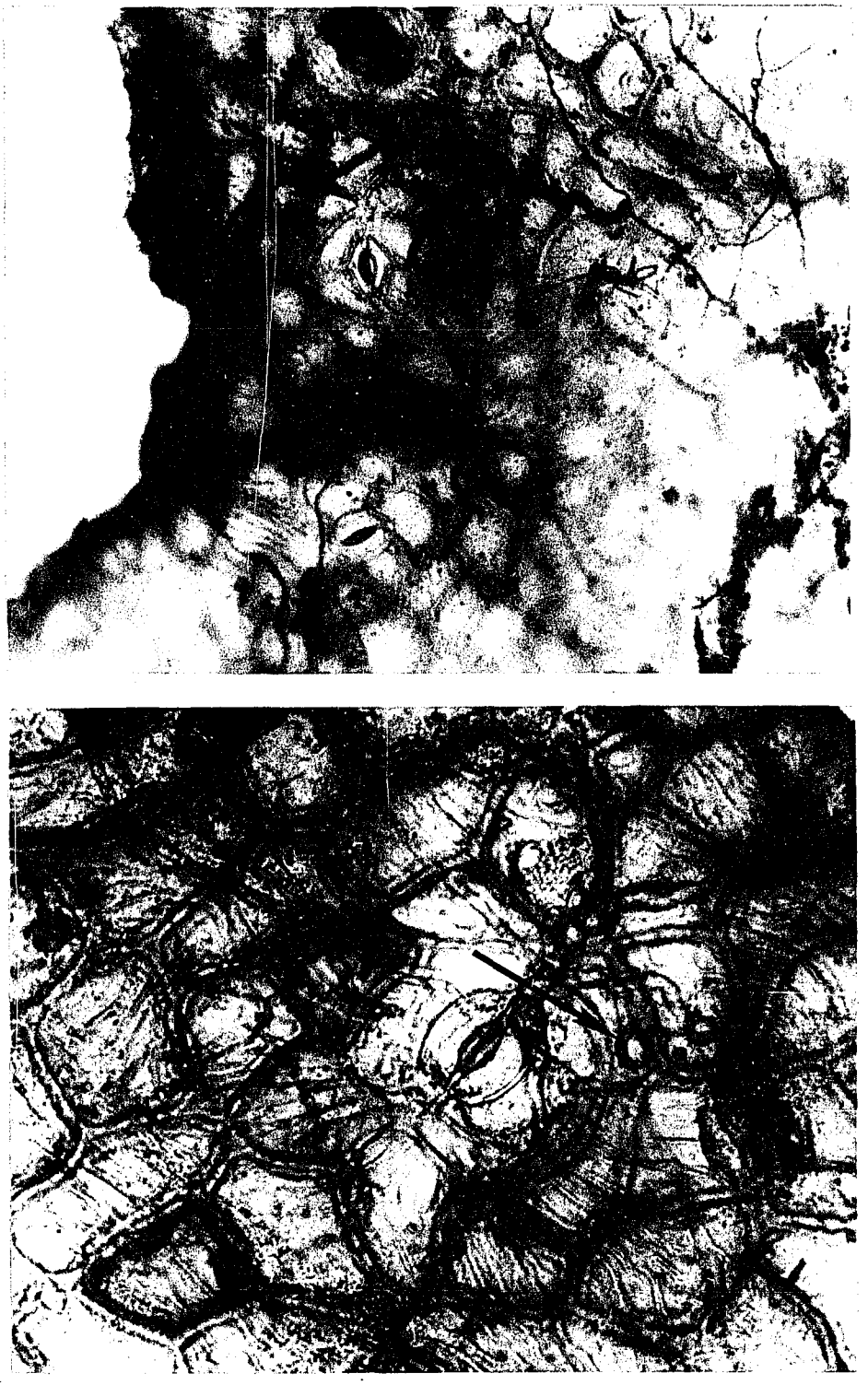


Figure 27. Paradermal sections of Diplodia surface inoculated greenhouse bolls. Upper 10-day boll surface (210X) and lower 20-day boll surface showing chlamydospore formation (535X). Note closed stomata.

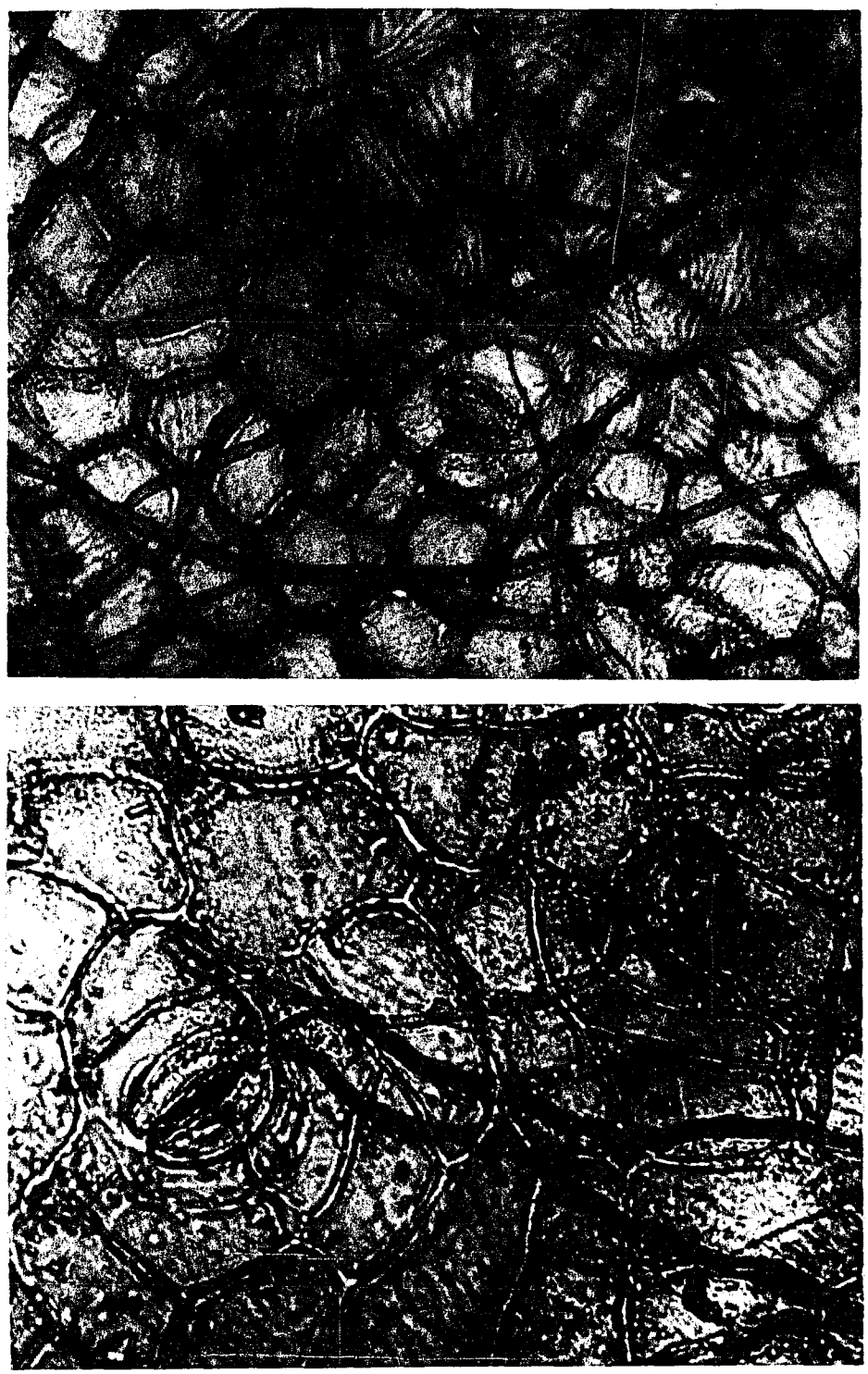


Figure 28. Paradermal sections of Diplodia surface inoculated greenhouse bolls. Upper 30-day boll surface with branching hyphae on wrinkled cuticle with closed stoma (430X) and lower stomatal penetration of 40-day boll (645X).

Figure 29. Paradermal section of Diplodia surface inoculated greenhouse 50-day boll. Note germinated spore on wrinkled cuticle and completely opened stoma. 750X

Transverse sections of boll carpel walls, ages 5-60 days, also exhibited abundant boll stomatal penetration and growth within the epidermal layers (Figs. 30, 31, 32, 33).

The confinement of the Diplodia hyphae to the epidermal layers of the boll compares quite favorably to that seen for the field bolls. However, the progress of boll maturity and fungal development further into the boll tissues was extended some 15-20 days in the greenhouse bolls (Figs. 31, 32, 33).

It was also noted that, while the Diplodia mycelium colonized the epidermal layer, hyphae could also be found along the outer periphery of the cells comprising the stomatal chambers. At this point pycnidial formation proceeded on the guard cells.

These processes took place long before the actual penetration of the locules by hyphae from which boll rot developed.

Fungal growth proceeded to the outer limits of the mesocarp tissue and was later observed entering the dehiscing sutures (Fig. 33). As in the field bolls, Diplodia hyphae could be found colonizing the sloughing sutural line parenchyma and advancing down the dehiscing suture to the locule. Fiber rot occurred before external boll dehiscence was complete.

Diplodia mycelium was seen to accumulate on the external boll sutures at the cuticle. This made fungal inoculum available for sutural penetration at the time of boll dehiscence (Fig. 34).

Greenhouse Bract Studies:

The mode of entry and colonization of bracts by D. gossypina was determined. Fungal spore suspensions were applied to greenhouse

Figure 30. Transverse section of Diplodia surface inoculated greenhouse 45-day boll showing fungal penetration of a stoma with hyphal tips extending into the substomatal chamber. 645X

Figure 31. Transverse sections of Diplodia gossypina Cke. surface inoculated greenhouse bolls showing intercellular fungal growth beneath the boll epidermis. Upper 45-day boll (500X) and lower 50-day boll (535X).

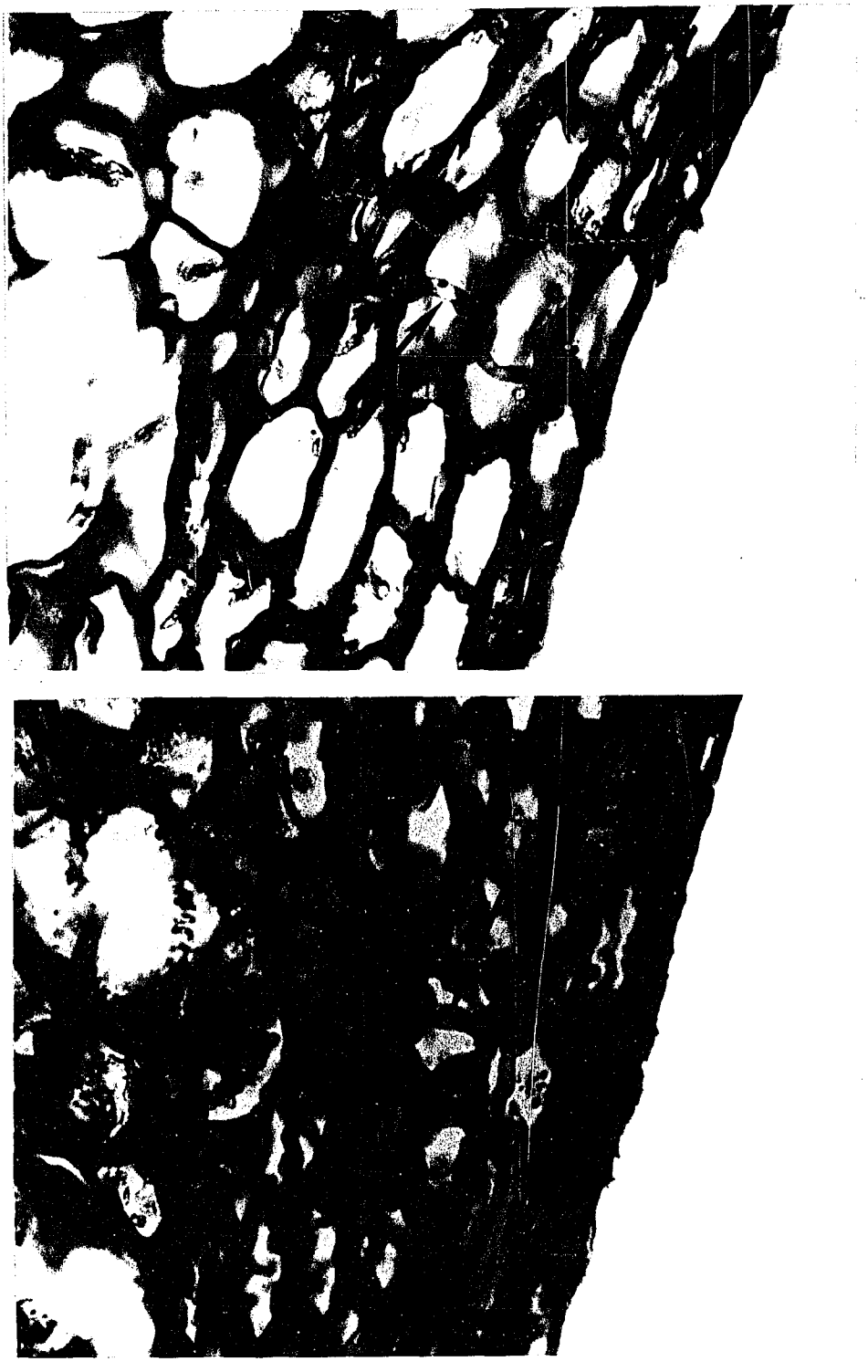


Figure 32. Transverse sections of Diplodia surface inoculated greenhouse bolls showing intercellular fungal growth beneath the boll epidermis. Upper 55-day boll (500X) and lower 60-day boll (430X).

Figure 33. Transverse sections of dehiscing greenhouse bolls, surface inoculated with Diplodia, exhibiting fungal penetration of the sutural area. Upper 45-day boll (320X) and lower 55-day (210X).

Figure 34. Transverse section 50-day greenhouse boll surface inoculated with Diplodia. Note typical accumulation of mycelium at the sutural area. 430X

bracts as they first appeared on squares and on through dehiscence. The time interval indicating bract stages was 5 days. The plants were grown under similar humid conditions described for the inoculated bolls.

After five days, 4 bracts from each age interval were removed and individually surface sterilized in 95% ethyl alcohol for 1-2 min and then in 1:1000 mercuric chloride for 30 min. They were rinsed in sterile water and sections were taken aseptically from the apex, middle, and base of each bract to be plated on water agar. The bract position from within which microbial growth developed was recorded in Table 6.

Fungal infection of all of the bract apices was observed. The middles and bases of the bracts seemed virtually devoid of infection at the later square and early boll stages.

The squares of the 1 to 10-day periods appeared to be severely affected by the fungal infections as a result of the spore suspension inoculations. Almost all of the 1 to 5-day inoculated squares dropped from the plants, and only a few 10-day squares remained attached to the plants. Isolations made from the fallen squares revealed D. gossypina within the square tissues. No distinction was made as to the fungal progression within the internal bract tissues of the 1 to 5-day squares. The bract tissue breakdown occurred very shortly after the squares dropped from the plant. This square loss was not observed in the other half of the same greenhouse bench which was held as a check and not inoculated.

Table 6. Developmental progression of Diplodia gossypina Cke. within surface inoculated bracts of stated ages.

Age (days) of inoculated bracts/1	Bract portions showing microbial growth/2		
	Apex of bract	Middle of bract	Base of bract
1	+	+	+ /3
5	+	+	+ /3
10	+	+	
15	+		
20	+		
(anthesis) 0	+		
5	+		
10	+		
15	+	+	
20	+	+	
25	+	+	
30	+	+	
35	+	+	+
40	+	+	+
45	+	+	+
50	+	+	+
55	+	+	+
60	+	+	+

/1 Four whole bracts selected for each age interval.
/2 Removal of inoculated bracts from bolls after 5 days.
/3 Entire inoculated squares exhibited rot and fell from plant.

At the 35-day boll period, all of the bract portions produced fungal growth and were considered completely infected.

Visual evidence of D. gossypina hyphae within greenhouse bracts was produced by paradermal sectioning of previously inoculated boll bracts aged 10, 20, 30, 40 and 50-days.

Intercellular hyphae seemed to be confined to the bract apex of the 10-day period (Fig. 35 upper). The twenty and thirty day bracts were invaded to their middle portions (Figs. 35 lower, 36, 37 upper). Complete bract infection was seen in the 40 to 50-day bracts (Figs. 37 lower, 38, 39, 40).

Bract apical tissue began to turn yellow-brown near the 40th day of development on the boll. Apical decay was observed after the 40th day and progressive bract deterioration until boll dehiscence at 65-75 days.

Initial bract penetration by D. gossypina appeared to be through the stomata of bract apices (Fig. 41). The stomata (hydathodes) of the bract margins were principally involved, although the interior stomata were sometimes found to have been penetrated.

Figure 35. Paradermal sections of greenhouse bracts, surface inoculated with Diplodia, showing intercellular growth of hyphae. Upper 10-day bract apex and lower 20-day bract apex. 750X

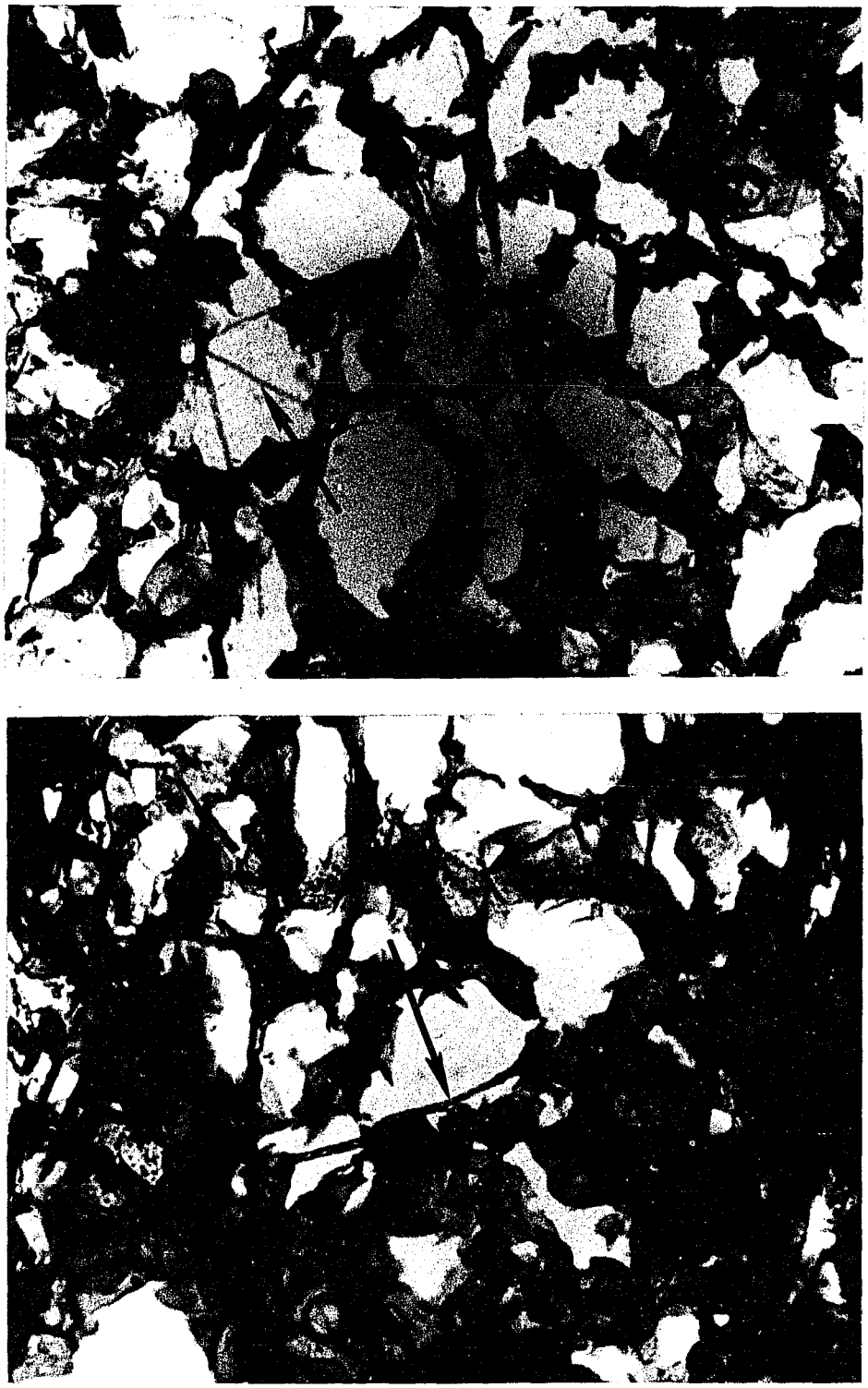


Figure 36. Paradermal sections of greenhouse bracts, surface inoculated with Diplodia, showing intercellular growth of hyphae. Upper 20-day bract middle and lower 30-day bract apex. 645X

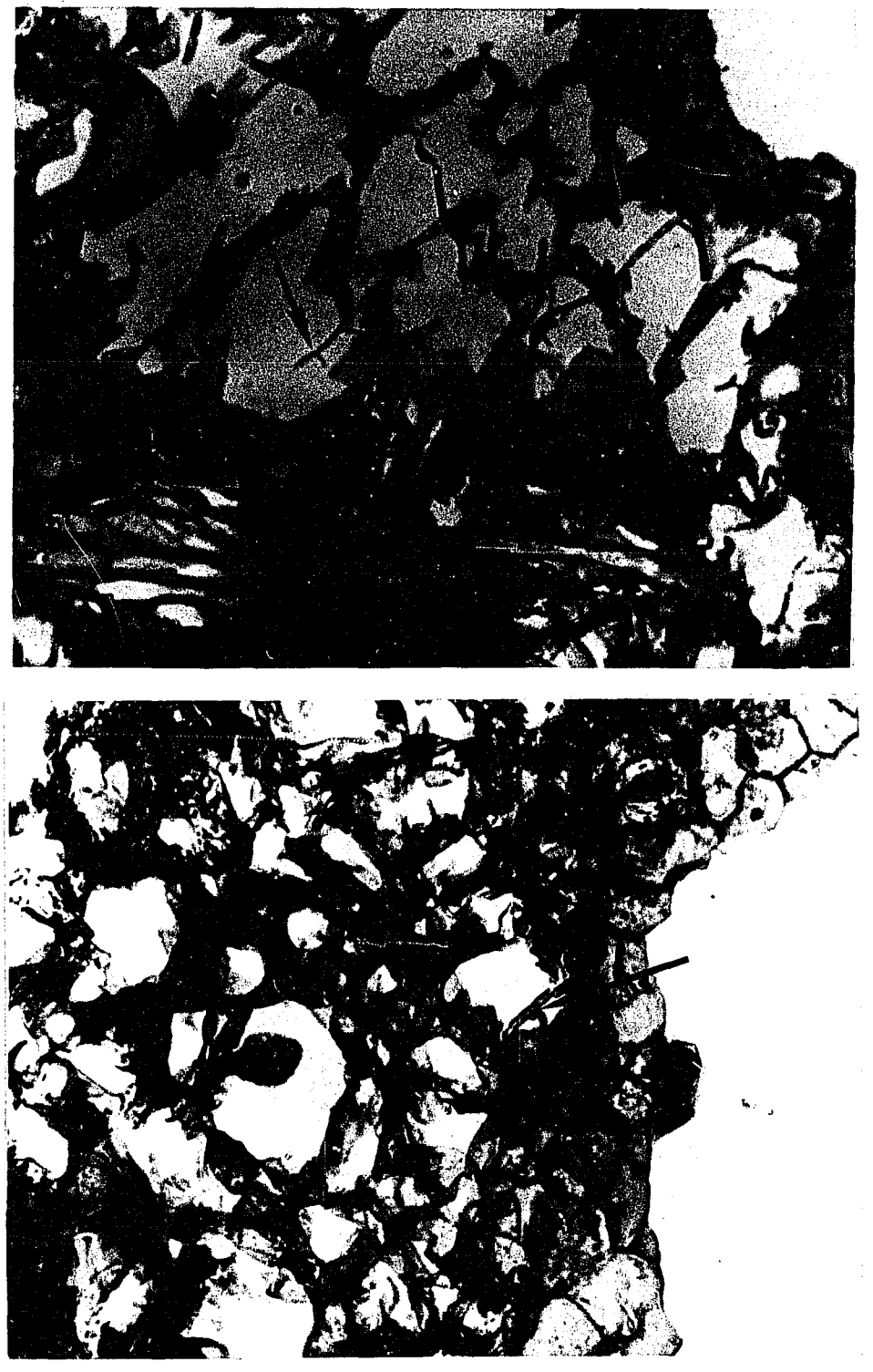


Figure 37. Paradermal sections of greenhouse bracts, surface inoculated with Diplodia, showing intercellular growth of hyphae. Upper 30-day bract middle (750X) and lower 40-day bract apex (645X).

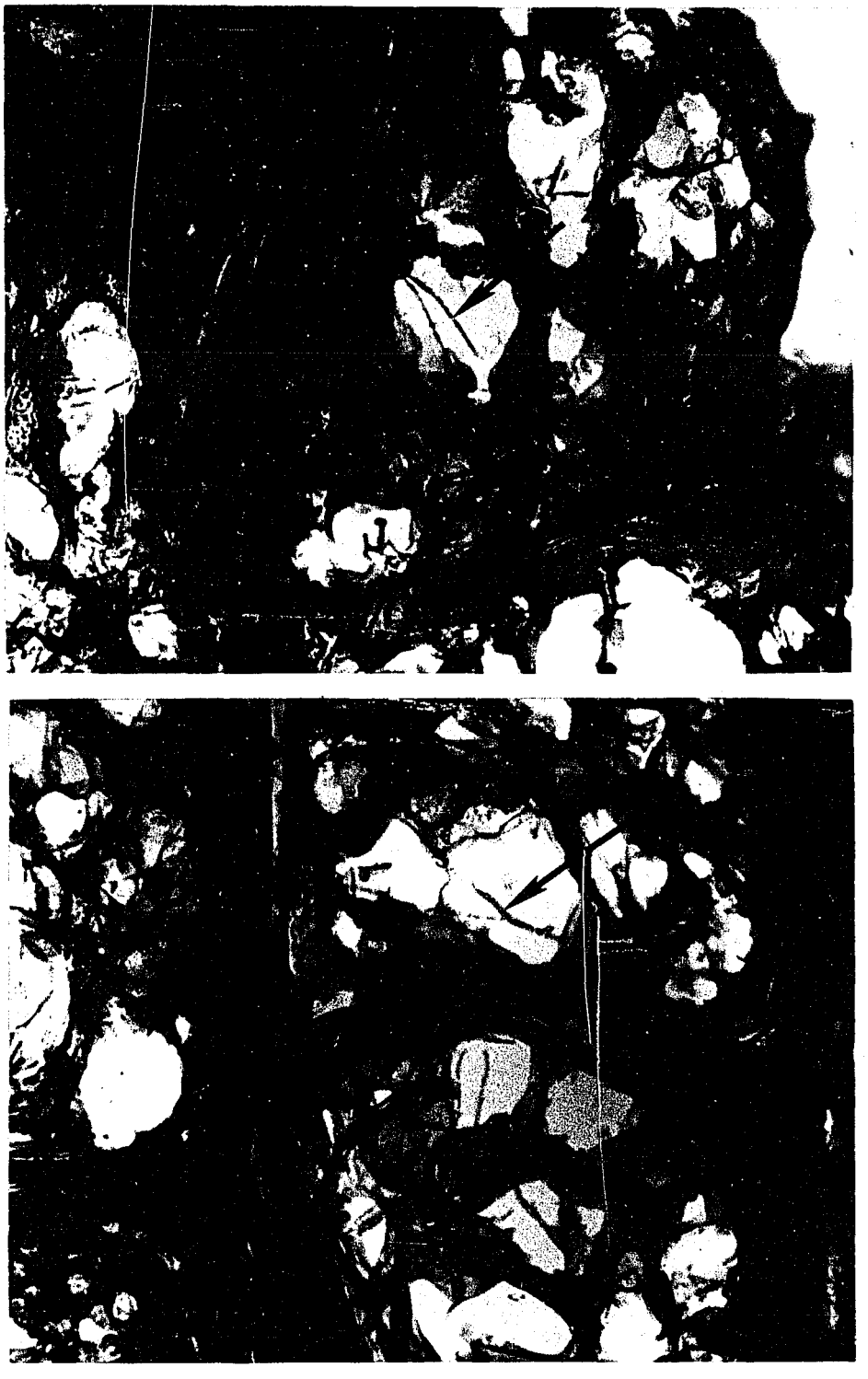


Figure 38. Paradermal sections of greenhouse bracts, surface inoculated with Diplodia, showing intercellular growth of hyphae. Upper 40-day bract middle (645X) and lower 40-day bract base (750X).

Figure 39. Paradermal sections of greenhouse bracts, surface inoculated with Diplodia, showing intercellular growth of hyphae. Upper 50-day bract apex (645X) and lower 50-day bract middle (750X).

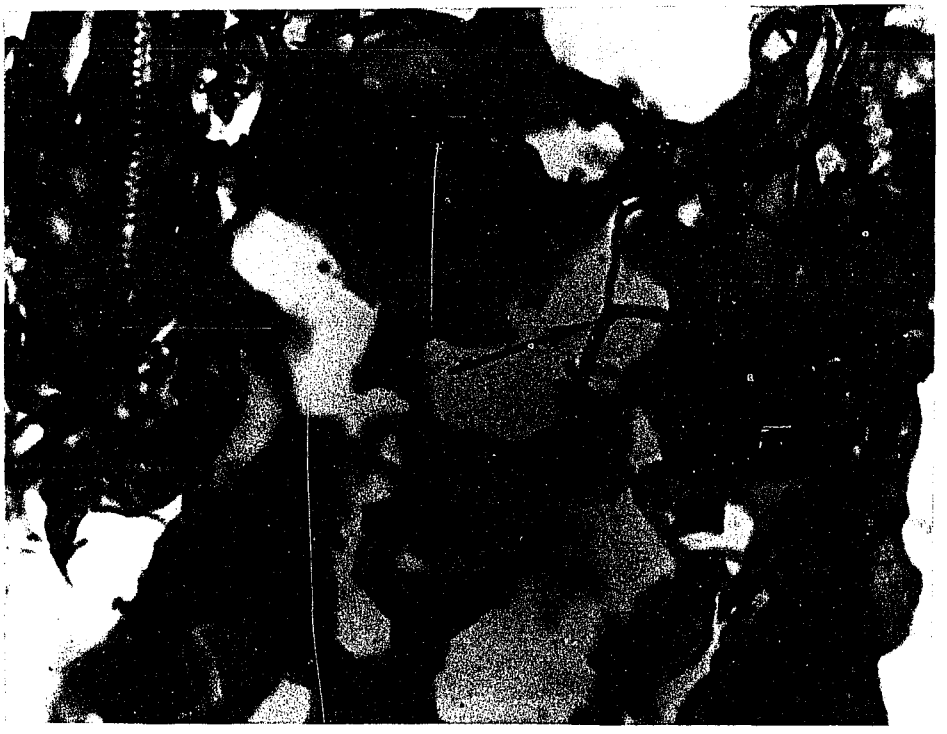


Figure 40. Paradermal section of 50-day greenhouse bract base showing intercellular growth of hyphae. 750X

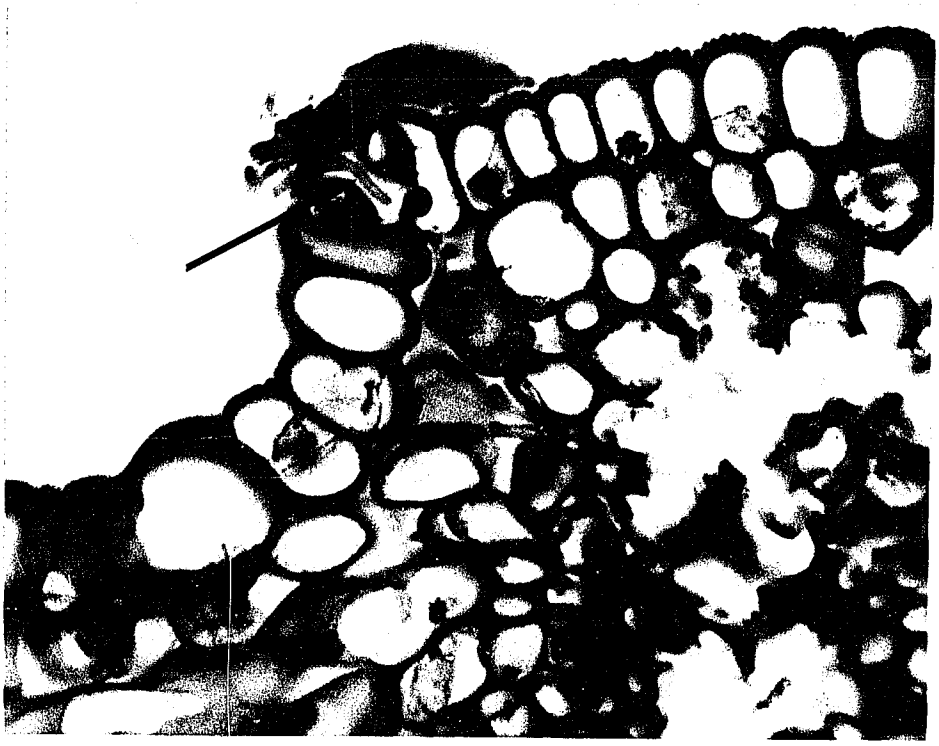


Figure 41. Transverse section of 35-day greenhouse bract apex, surface inoculated with Diplodia, indicating fungal penetration of a stoma. 430X

DISCUSSION

In the 1969 cotton growing season Louisiana experienced a very low percentage of boll rot, the state average being approximately 1-2 percent. This was mainly the result of very low amounts of seasonal rainfall and the usually high temperatures.

The Morrow and St. Joseph plots experienced low rainfall, the Morrow plot receiving slightly more precipitation early in the season, but both producing mature cotton by the 50th day from anthesis (Fig. 6).

Early season rains were probably responsible for the slightly higher level of microbial inoculum available for early bract and boll infection in the Morrow plot as opposed to the St. Joseph field as seen in Table 6 and Figures 18 and 19.

The unusually dry season had its effect on the development of the various boll-rotting organisms during the cotton season. The primary boll penetrators, such as Collectotrichum gossypii Southworth, Pellicularia filamentosa (Pat.) Rogers, Phytophthora parasitica Dast. and others, were not among those organisms isolated from Morrow or St. Joseph. The state survey produced only a few isolations of these groups and only from those areas receiving large amounts of precipitation through the season.

Those microorganisms which are considered as secondary pathogens were the major group responsible for boll rot. Tables 3 and 5 contain the microorganisms which were commonly found in the cotton boll-rot disease complex in 1969.

Bolls taken from the Morrow plot began centrifugal dehiscence between the 10th and 15th day from anthesis (Figs. 4 upper, 8). The exact region of initial dehiscence and tissues involved were the parenchyma of the sutural line located between the sclerenchyma fiber bundles (Fig. 8). This is the first report of the specific mode of splitting of the boll suture and the fact that dehiscence begins before the boll reaches external maturity--about 20-25 days in 1969.

The knowledge of the method and time of initial boll dehiscence is important because it seems to coincide with the entry of the boll epidermal tissue by boll-rotting organisms (Fig. 10).

External boll suture anatomy was presented (Figs. 1, 2, 7, 8). The internal parenchyma ridge, a previously overlooked structure, was identified (Figs. 1B, 2H, 7B). The importance of this parenchyma structure is its continuous connection with the boll suture line to the epidermis. The "ridge" represents one of the few weak points in the otherwise continuous sclerotized endocarp of the carpel (Figs. 1, 2, 7).

The sclerenchyma fibers of the endocarp present a physical barrier to secondary boll pathogens and possibly primary invaders as well. The non-sclerotized zone, created by the internal parenchyma ridge interrupting the endocarp, appears to be a natural avenue of entry into the boll locule.

Boll stomata were seen as the primary mode of microbial penetration into cotton bolls taken from both the Morrow and St. Joseph plots (Figs. 10 upper, 11 lower, 12, 13, 14).

The paradermal sections of field bolls ages 5-45 days revealed mycelia of various species of fungi randomly covering the cuticle and/or entering boll stomata. Hyphae invaded the epidermal layers and growth appeared as intercellular (Fig. 11) and intracellular (Fig. 10 lower) colonization.

Field boll guard cells ceased to function between the 15th and 20th day of boll maturation, and the stomata remained opened in 1969. Fungal penetration of stomata followed guard cell senescence.

The cotton variety 'DPL 16' produced 15-20 boll stomata per 100X microscope field. This adequate number of stomata, while allowing efficient gaseous exchange between the boll tissues and its external environment, also provided numerous points of entry for microorganisms such as bacteria and fungi.

Fungal growth was confined to the epidermal layers during early boll dehiscence (Figs. 15, 16 upper) in 1969. Hyphal penetration proceeded to the outer mesocarp tissue and into the dehiscing boll sutures (Figs. 16 lower, 17).

The principal source of fungal nourishment within the dehiscing sutures appeared to be the sloughing parenchyma cells of the sutural line (Fig. 17). These cells were utilized by the fungi until the boll suture had dehisced through the internal parenchyma ridge, exposing the locule and fiber.

Locule penetration and fiber rot took place at different rates depending on the level of pathogenicity of the microorganisms. Diplodia gossypina Cke. entered the locule and rotted the fiber before the suture had completely dehisced externally. Boll fiber infected

with Fusarium sp. began to rot at boll dehiscence; however, Alternaria tenuis Nees rotted the fiber only after the bolls were completely dehisced, exposing the fiber within.

The activity of microorganisms within the boll pericarp preceding complete sutural opening was considered as latent infection.

Microbial presence within the pericarp of field bolls following guard cell senescence (Table 1) may account for isolations, by some workers, of various bacteria and fungi from excised and incubated apparently healthy field bolls (1, 2, 3, 5, 17, 18). The methods of boll surface sterilization employed by these researchers were observed to have little or no effect on microbial growth within substomatal cavities and associated tissues as seen in Figures 18 and 19. Many of the microorganisms, isolated from various boll regions other than the pericarp by these workers, were taken primarily from the tissues of the pericarps in this study (Tables 2 and 3).

No rot developed in 17 Morrow and 13 St. Joseph bolls in the 5-20 day age range on which callus tissue was formed on the cut surface (Fig. 20). This production of callus tissue would seem to indicate that the tissues of young bolls are comparatively free of microbial growth.

An increase in rotted bolls was observed after the 15th-20th day (Fig. 18), and only 3 bolls had callus tissue after that period. This information seemed to agree with the time of boll stomatal penetration and colonization of the epidermal tissue (Figs. 10-16).

Decrease in callus tissue production following the 20th day of boll age, appeared to be caused by the continuous maturation of the

tissues comprising the bolls. Most of the tissue regions showed thickening of cell walls which usually precedes reduction of meristematic activity.

Microorganisms gaining entrance into the pericarp may perish before they can get to the sutural parenchyma if the boll matures quickly as a result of hot, dry weather. The reverse may enhance infection because the boll tends to remain closed during wet weather.

It was noted that, while the pericarp tissue yielded abundant microbial growth, the placental column tissues contained no evidence of microbial occurrence until the bolls were nearly dehisced at 40-45 days from anthesis. The observation of apparently disease-free placental columns in the early boll stages agreed with results presented by Guidroz and Pinckard (9).

Apparently healthy field bracts from all stages of floral development through to fruit dehiscence were shown to contain various species of microorganisms on and within their tissues. This was shown to be a major source of potential boll-rot inoculum which increased in the early floral and boll stages (Table 5). The inoculum was available for boll stomatal penetration at the 15 to 20-day period.

Bracts in all stages of development were thoroughly surface sterilized and individually sectioned into apical, middle, and basal portions. These portions were placed on water agar and incubated. Bract apices taken from boll stages produced abundant microbial growth (Table 4). This was also shown by Luke (12) and Delgado (6). Further infection of bract middles and bases, however, was also observed. In addition, this study also established for the first

time the presence of microorganisms within the bracts on all square stages (Tables 4 and 5).

Alternaria tenuis Nees was the predominant bract invader from the very earliest bracts on squares to complete boll dehiscence (Table 5). It was shown that a large number of the microorganisms isolated from field bracts were also associated with the external and internal regions of field bolls (Tables 2 and 3).

Visual presence of microorganisms within field bract portions supported the findings of the microbial isolation work.

Paradermal and transverse sections of bracts 5-45 days from anthesis revealed hyphae of various fungal species colonizing the bract tissues intercellularly (Figs. 22, 23, 24, 25, 26). Tissue breakdown and thicker-walled hyphae were seen beginning in the apical portion of the bracts. These findings agree with those of Delgado (6).

Initial fungal penetration of the bracts occurred near their apices, principally through the hydathodes, in accordance with Delgado (6); however, bract entry was also accomplished through the normal surface stomata of the bract apex (Fig. 22).

The mode of penetration and colonization of Diplodia gossypina Cke. in greenhouse bolls was demonstrated. Bolls of age 5-60 days (5-day intervals) were surface inoculated with spore suspensions from a field isolate of D. gossypina. High humidity was maintained to insure maximum fungal activity on the boll surfaces. The inoculated bolls were removed at the end of 5 days. Paradermal and transverse sections of the boll pericarps were prepared.

Paradermal sections showed fungal growth on practically all of the boll surfaces (Figs. 27, 28, 29), and penetration of bolls through the stomata was observed (Figs. 28, 29, 30). This agreed with Delgado's findings (6).

The ability of microorganisms to persist on the boll surfaces until they enter the boll has been questioned (5, 20). The evidence gathered, indicating abundant microbial populations inhabiting the field and greenhouse boll surfaces, points up this ability of microorganisms to sustain themselves while confined to the boll cuticle.

Many of the fungi produced dark pigmented mycelium which would reduce the detrimental effects of sunlight on protoplasm (Figs. 9 upper, 11 lower, 27, 28, 29). Thick-walled spores and/or mycelium (chlamydospores) were seen as another possible reason for survival (Figs. 9 upper, 27 lower, 29).

Deep striations in the cuticle and indented sutures afforded sites of attachment and possible moisture conservation for hyphae located in them (Figs. 28 upper, 29, 34). Guard cell senescence permitted stomatal opening and the release of moisture and possible sugars of various types to the exterior of the bolls where the latter could be assimilated by surface inhabiting microorganisms.

The time of guard cell senescence and stomata penetration in greenhouse bolls was lengthened over that of field boll stomata (Fig. 28 lower). The guard cells ceased functioning between the 35th and 40th day, and fungal penetration followed. These results differed from Delgado's observations (6), which were made under less humid circumstances.

The optimum conditions of adequate moisture, fertilizer, and the "greenhouse effect" to which the bolls were subjected prolonged boll tissue maturation and dehiscence (Fig. 33) and contrasted with the dry season of 1969.

D. gossypina readily penetrated the boll stomata and grew within the epidermal layers during the 30-40 day periods of boll tissue development (Figs. 28 lower, 29, 30, 31, 32). Hyphae were observed in the region of and entering the dehiscing boll sutures by the 40th-45th day (Fig. 33 upper). Growth proceeded through the suture to the locule as dehiscence continued (Fig. 33 lower). Fiber rot was observed to develop before external dehiscence in several instances.

Results showing the progressive development of D. gossypina within greenhouse boll pericarp tissues and the subsequent rotting of the fiber in the locule support the information obtained from the field boll studies.

Observations of Diplodia surface-inoculated greenhouse bracts on squares and bolls through dehiscence compared favorably to those of the field bract studies.

Fungal growth from bract apices at all age intervals was seen. This compares to results recorded by Delgado (6) and Luke (12), although they did not study bracts on squares. The middle and base of the variously aged bracts did not produce fungal growth, however, until the 15th day and 35th day, respectively, with the exception of those bract portions on the 1 to 10-day square stages which were

seriously affected by infection of Diplodia (Table 5). The squares of the 1 to 5-day stages exhibited rot and fell from the plants, an observation in agreement with Sciumbato (20). Only a few 10-day squares remained attached to the plants. Square loss as a result of inoculation was not observed in the plants of the other half of the same bench which was held as a check.

For the first time, visual evidence of fungal entry and colonization of the entire bract tissue was presented and it verified the results of the isolation studies. Intercellular hyphae were seen only in the apices of the 10-day boll bracts (Fig. 35 upper). Further fungal progression within the bract tissue was noted in the 20-day bract sections (Figs. 35 lower, 36 upper) and continued to the bract bases by the 40-day stage (Fig. 38). Complete bract breakdown was observed in the 50th-day bract sections (Figs. 39, 40).

Bract entry by D. gossypina was through the stomata (Fig. 41), a finding which agreed with the field bract work (Fig. 22).

The results obtained from these studies involving the mode of boll dehiscence and the penetration of bolls and bract tissues by microorganisms have suggested projects for future study.

The intimate association of the internal parenchyma ridge with the boll fiber in the locule may be shown to be a possible link between the rapidly maturing fiber and boll dehiscence. It may be possible to demonstrate a release of some stimulatory compound, such as an enzyme, by the cotton fiber which would be absorbed by the parenchyma of the "ridge" and transported to the region of initial sutural dehiscence.

Another study might be made to determine the exact role of the bacteria, other than those considered as primary pathogens, in predisposing the boll tissues to rot by pathogens of a higher level, such as fungi. Boll and bract isolations made during the course of studying those microorganisms involved in boll rot in 1969 and their mode of entry into field boll tissues revealed a large number of various species of weak bacterial boll pathogens. This observation was in accord with isolation studies from previous years which showed various bacterial types, which were thought to be contaminants, combined in mixed cultures with fungi taken from rotting field bolls.

A study conducted to remove all doubt as to what is meant by the term "direct boll penetrator" is seriously needed. Prepared microtome sections should be made of bolls surface inoculated with the so-called "primary or direct penetrators." These sections should reveal the pathogens directly penetrating the cuticle of the boll--not the stomata--and, likewise, their entrance to the locule through the endocarp tissue--not through the sutural line.

SUMMARY

1. Field grown bolls began centrifugal dehiscence between the 10th and 15th day from anthesis during the dry cotton growing season of 1969.
2. The tissue involved in initial boll dehiscence was the parenchyma of the sutural line, located between the sclerenchyma fiber bundles.
3. The internal parenchyma ridge, a parenchymatous structure which extended from the apex to the base of the boll and into the locule, was identified. This "ridge" was found to play a significant role in boll rot initiation.
4. The primary mode of microbial penetration into Morrow and St. Joseph bolls in 1969 was through the stomata, which remained open on bolls after age 20-25 days from anthesis.
5. Surface sterilization of field bolls did not effect the microbial growth within the boll pericarps. Microorganisms which are confined to the pericarp during boll maturation constitute latent infection.
6. Boll rot in the Morrow and St. Joseph plots in 1969 was caused primarily by secondary boll pathogens which penetrate the boll stomata and proceed through the dehiscing sutures to the locules.
7. Field bolls which were surface sterilized and incubated did not exhibit significant rot prior to the 20-day boll stage, and callus tissue formed on many of the 5 to 20-day bolls.

8. Microorganisms were not isolated from the boll placental columns until dehiscence was almost complete at 40-45 days.
9. Microbial infection of bracts on squares of all ages was shown for the first time.
10. Alternaria tenuis Nees was the primary bract invader in the Morrow and St. Joseph plots. The fungus was isolated from all stages of bract development.
11. Microorganisms isolated from field bracts were essentially the same as those taken from field boll tissues and rotted bolls.
12. Visual presence of intercellular microbial growth within all portions of bracts was revealed.
13. Observations of Diplodia gossypina Cke. penetrations and colonizations of greenhouse-grown bolls and bracts were similar to those of the field studies, except for delayed tissue maturity in greenhouse bolls.

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VITA

Lloyd Frederick Baehr, Jr. was born at New Orleans, Louisiana, January 17, 1938. He graduated from East Jefferson High School, Metairie, Louisiana, in 1956. He entered Auburn University in September, 1956, on a football schlorship while pursuing a B.S. degree in Agricultural Engineering. In January, 1958, he attended Tulane University in Mechanical Engineering. He returned to the field of agriculture while enrolled at Southeastern Louisiana College in September, 1959, where his Bachelor degree was obtained in May, 1961. He entered the Graduate School of Louisiana State University in September, 1961, and received a teaching assistantship and then a research assistantship in Plant Pathology. He received the degree of Master of Science in January, 1964. He resigned in September, 1964, to accept a teaching position in Botany at Louisiana State University in New Orleans where he was employed until September, 1966.

He worked as a marine surveyor, for Superintendence Co., New Orleans, Louisiana, supervising the loading of grain aboard ships destined for export sales. In February, 1967, he joined the United States Department of Agriculture, Division of Plant Quarantine, in New York, New York and then Miami, Florida where he served as a GS-9 journeyman inspector.

He reentered the Graduate School of Louisiana State University in September, 1968, and received a research assistantship in Plant Pathology. He is now a candidate for the degree of Doctor of Philosophy in August, 1970.

He married Dianne Marie Leininger in 1960, and has five children.

EXAMINATION AND THESIS REPORT

Candidate: Lloyd Frederick Baehr, Jr.

Major Field: Plant Pathology

Title of Thesis: Histological Studies on the Mode of Penetration of Boll-Rotting Organisms into Developing Cotton Bolls

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

July 20, 1970